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Studies on the Molecular Cloning of a Gene for Aryletherase From a Ligninolytic Erwinia Sp. (Lignin, Biodegradation, Bacterial).

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STUDIES ON THE MOLECULAR CLONING OF A GENE FOR ARYLETHERASE FROM A LIGNINOLYTIC *ERWINIA* SP.

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ABSTRACT

Recombinant DNA techniques were utilized to clone a gene from the ligninolytic system of a saprophytic Erwinia sp. into Escherichia coli using pBR322 as a vector. The cloned gene encodes aryletherase activity and was selected from amp^r tet^S transformants of E. coli Cs412 using salicin as the selective carbon source. This compound has structural characteristics similar to some lignin model compounds. The cloned gene encodes activity which oxidizes aromatic lignin model compounds resulting ultimately in the breakage of arylether bonds while leaving the aromatic nucleus intact. At least one lower molecular weight aromatic compound is also released from kraft lignin upon incubation with whole cells harboring the recombinant plasmid, pNC1. Restriction enzyme analysis revealed that pNC1 contains an insert of approximately 810 bp of Erwinia sp. DNA. Southern blot hybridizations indicate that the gene is present as a single copy on the Erwinia sp. genome. Dideoxynucleotide DNA sequencing suggests that the gene is transcribed by readthrough of transcription initiated at the tetracycline resistance promoter in pNC1. The predicted translation product has an estimated molecular weight of 21,000 daltons.

INTRODUCTION

The increasing costs of chemical feedstocks produced from petroleum has led to a number of investigations aimed at the development of processes which utilize renewable resources for these purposes. Much attention has focused on the utilization of lignocellulosics as primary sources of raw materials. Next to cellulose, lignin is the second most abundant organic polymer on earth, comprising about one-fourth of the wood tissue in plants. Lignin is also the major solid component of the spent sulfite liquor of the pulp and paper industry, and in this form presents a considerable waste problem.

Development of efficient microbial biodegradation of lignin could lead to technological advances toward the production of valuable chemicals from native lignin and lignin wastes. At present, there is no industrial process for the bioconversion of lignin into useful products.

The biochemical basis for lignin degradation has been poorly understood, and only recently have enzymes actually been shown to be involved in the degradative processes (20, 39, 90, 91).

During recent investigations in our laboratory, a bacterium capable of growing on kraft lignin as the sole carbon source was isolated and tentatively identified as an Erwinia sp. During lignin degradation lower molecular weight intermediates are released from the lignin polymer. The organism has also been shown to oxidatively metabolize lignin model compounds with arylether bond cleavage as part of the degradative process.

In the present study, molecular cloning experiments are described in which a gene encoding aryletherase activity was excised from the

Erwinia sp. genome and introduced into Escherichia coli Cs412 using pBR322 as a vector. E. coli clones carrying the recombinant plasmid encoding aryletherase activity, designated pNC1, were shown to release at least one lower molecular weight aromatic compound from kraft lignin. The clones also metabolize aromatic lignin model compounds by oxidation and effect ether bond cleavage without further ring cleavage. The gene encoding the aryletherase activity has been characterized at the DNA level. This cloned gene may prove valuable in the development of ligninolytic bioprocesses.

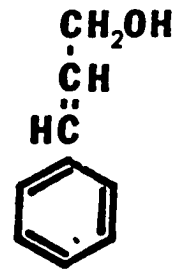
LITERATURE REVIEW

I. Distribution, biosynthesis, and structure of lignin.

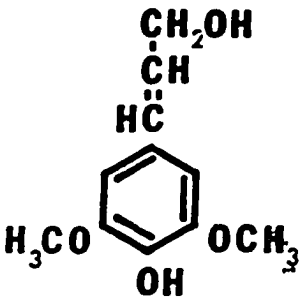
Next to cellulose, lignin is the second most abundant organic polymer in nature, accounting for 20-30% of the dry weight of vascular plants (85). It occurs as an integral cell component in the middle lamellae of vascular plants, where it functions as a cell cement (84). Lignin imparts structural rigidity with elasticity to plant tissues, minimizes water permeation across the cell walls of xylem tissue, and affords protection against infection by plant pathogens (84, 94). Lignin demonstrates a pronounced recalcitrance to biological degradation; its existence in close association with cellulose and other polysaccharides in plant tissues limits the microbial utilization of these compounds.

Structurally, lignin is a complex polymer of phenylpropane monomer units. Three cinnamyl alcohol derivatives, p-coumaryl, coniferyl, and sinapyl alcohols (Figure 1), are the immediate biosynthetic precursors of the lignin polymer (37). These alcohol subunits are present in different ratios in lignins from different plant species and often in different tissues of the same plant (7, 85). These alcohols are synthesized from aromatic amino acids as shown in Figure 2. Synthesis of phenylalanine and tyrosine proceeds via the shikimate pathway. Deamination of phenylalanine by phenylalanine ammonia lyase yields cinnamic acid which is then substituted in a sequence of hydroxylation and methylation steps (the cinnamate pathway). In some grasses tyrosine is deaminated to yield p-coumarate, an intermediate of the cinnamate pathway. The resulting cinnamate derivatives are subsequently reduced

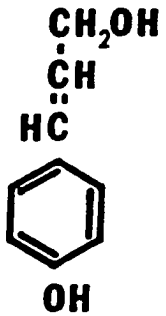
Figure 1. The biosynthetic precursors of lignin.



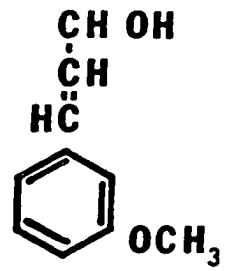
Cinnamyl Alcohol



Sinapyl Alcohol

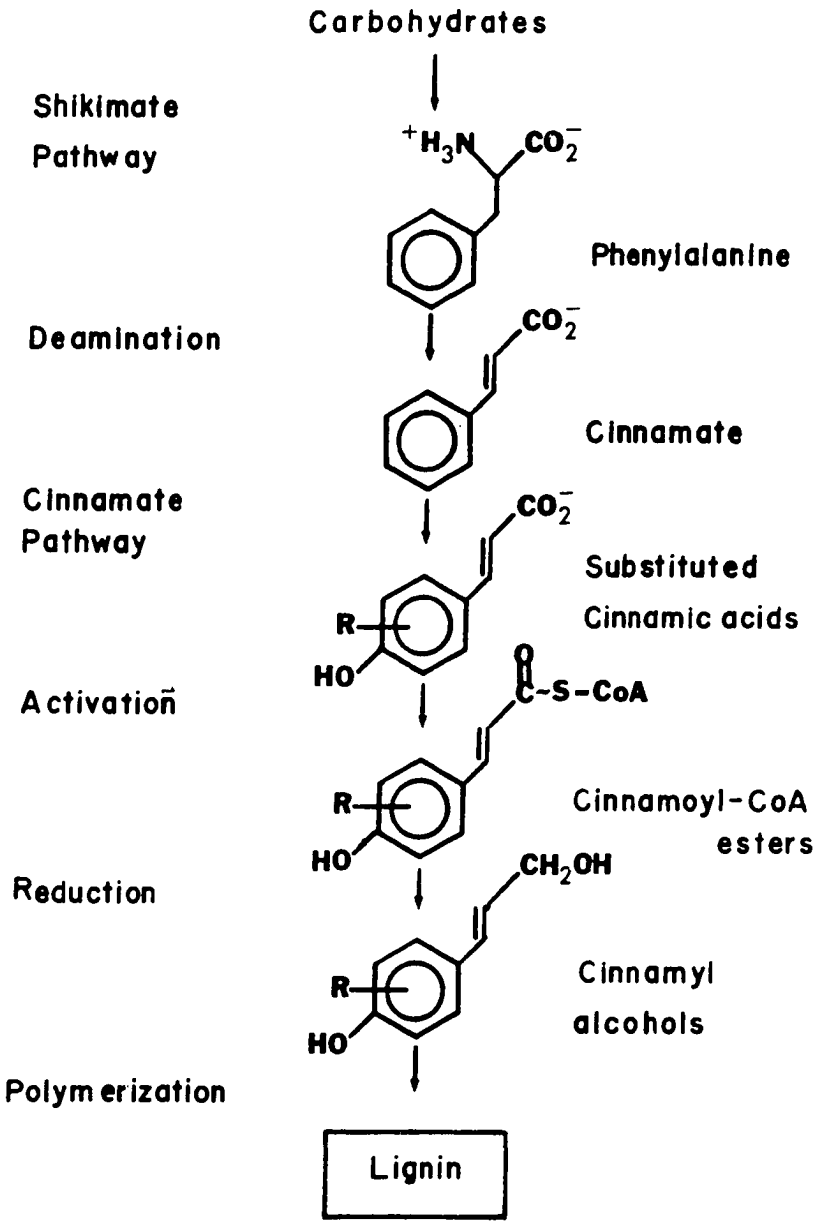


Coumaryl Alcohol



Coniferyl Alcohol

Figure 2. Schematic representation of the principal steps in lignin biosynthesis.



to the corresponding alcohols via CoA esters. Finally, these alcohols are polymerized to yield the lignin polymer (42, 49). Lignifying tissues possess the potential for the entire reaction sequence from phenylalanine to cinnamyl alcohols as the immediate lignin precursors (42). Thus, the suggestion by Freudenberg (38) that cinnamyl alcohols are synthesized in the cambium or phloem and transported in the form of glucosides into the xylem where, after release by β-glucosidase, they are polymerized to lignin is subject to question.

The lignin polymer is formed through the condensation and polymerization of phenoxy radicals derived from the three cinnamyl alcohol derivatives previously mentioned (37). Phenol oxidases produce free phenoxy radicals through the removal of an electron from a phenoxyl anion. The extended electron system of the molecules allows for stabilization of the free radicals through equilibrium with several mesomeric forms. Radical formation of coniferyl alcohol is given as an example in Figure 3. The phenolic hydroxyl groups in the growing lignin polymer are also subject to single electron oxidation and radical formation. Random condensation of all the mesomeric forms derived from coniferyl, sinapyl, and coumaryl alcohols with each other and with radicals in the growing polymer proceeds spontaneously.

Freudenberg (37) determined the presence of three major intermonomeric linkages: arylglycerol-β-arylether, phenylcoumaran, and biphenyl. Arylglycerol-β-arylether (30-50%) is the most prevalent linkage (85). As a result, lignin is a highly irregular, three-dimensional polymer with a number of nonrecurring C-C and C-O-C linkages that has no precise chemical structure and variable concentrations of monomeric units (Figure 4).

Figure 3. Formation of phenoxyradicals of coniferyl alcohol by phenol oxidase.

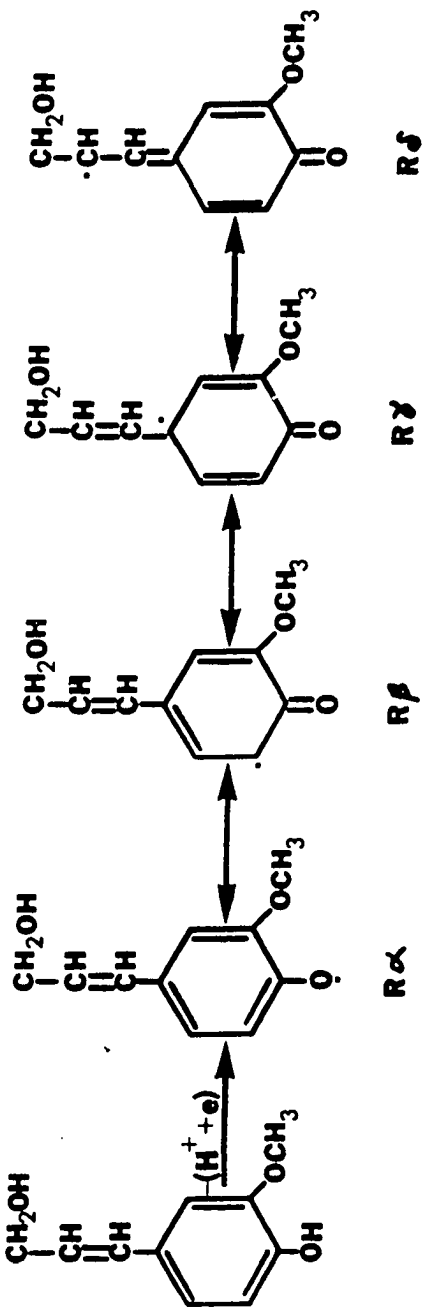
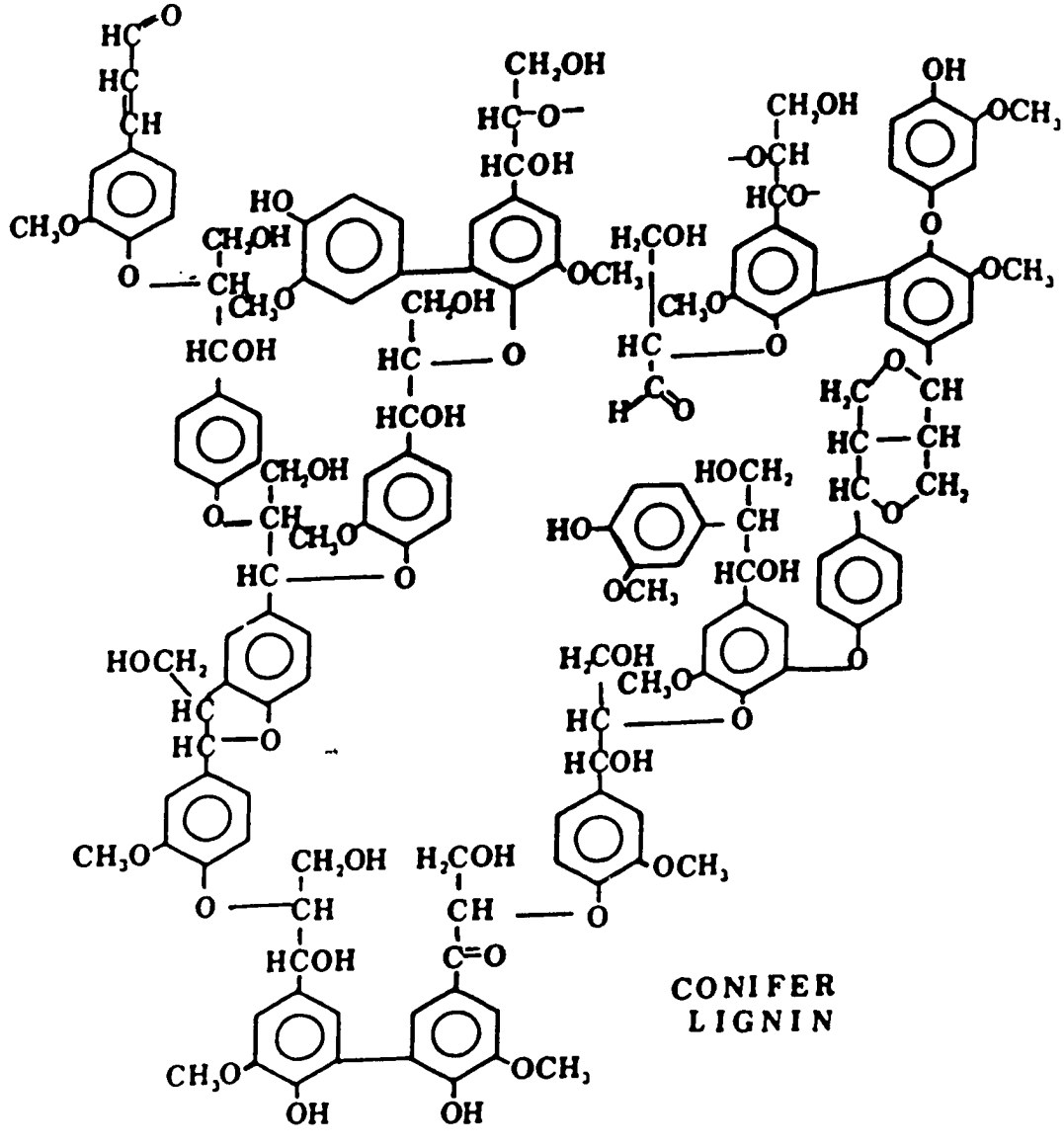


Figure 4. The structure of conifer lignin as proposed by Freudenberg (37).



CONIFER LIGNIN

Industrial lignins produced in the chemical pulping of wood for the manufacture of paper exhibit several structural alterations. Kraft cooking with alkaline sodium sulfide causes cleavage of arylether bonds between phenylpropane subunits which results in depolymerization of the three dimensional structure. Demethylation of methoxyl groups creating catechol moieties and strong modifications of the propane side chains to yield a variety of new structures also occur (70). The other major pulping process, acid sulfite cooking, results in the introduction of sulfonic acid groups in the α-position of the propane side chains and some aryl-alkyl ether cleavages between phenylpropane subunits (40).

II. Experimental approaches to the study of lignin biodegradation.

Early study of lignin biodegradation was hindered by the lack of a simple sensitive assay for the measurement of lignin decomposition. Recent advances in understanding lignin biodegradation have been made possible by the development of sensitive, specific radioisotipic methods for assaying lignin decomposition (for reviews see Crawford and Crawford (19) and Kirk, et al. (64). Two classes of radiolabeled substrates are used in tracer studies. ^{14}C-Lignin or ^{14}C-[Lignin]-lignocelluloses are natural substrates labeled specifically in their lignin component. ^{14}C-DHPs are synthetic dehydrogenative lignin polymers. Microbial degradation of ^{14}C-lignins can be monitored by following the conversion of substrate to $^{14}CO_2$.

^{14}C-[Lignin]-lignocelluloses are prepared by feeding the plant radioactive precursors such as ^{14}C-phenylalanine or ^{14}C-ferulic acid, followed by extraction of the lignocellulose (14, 19). ^{14}C-Lignins free of carbohydrate can be prepared by kraft pulping (16).

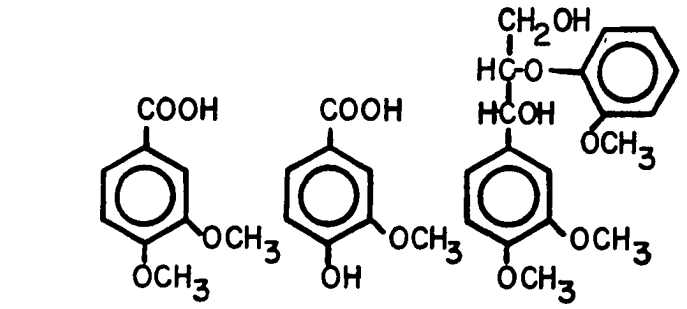
^{14}C-Synthetic lignins (DHPs) are prepared by the in vitro dehydrogenative polymerization of chemically synthesized ^{14}C-coniferyl alcohol in a peroxidase/H_2O_2 reaction (43, 62, 93).

^{14}C-[Lignin]-lignocelluloses are preferred substrates, in comparison with carbohydrate-free DHPs, for screening microorganisms that selectively delignify wood, for studying lignin degradation in natural ecosystems and for defining relationships between lignin and cellulose degradation in various microorganisms. In contrast, since DHPs do not contain carbohydrates or other extraneous materials they are ideal substrates for determination of an organism's ability to utilize lignin as a sole carbon source or for examination of cosubstrate metabolism (80).

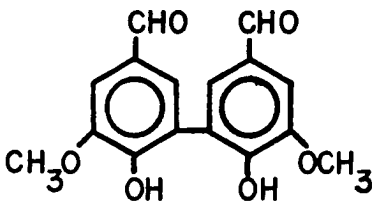
The structural complexity of the lignin polymer also makes elucidation of the biochemical mechanisms whereby microorganisms degrade lignin a difficult task. Simple, low molecular weight model compounds that contain chemical structures known to occur in lignin may be used to study the catabolic mechanisms of microbial decomposition of lignin.

Several types of compounds have been utilized as lignin models based upon chemical similarities with lignin substructures (Figure 5). Simple methoxylated aromatic acids (veratric acid, vanillic acid, and syringic acid), compounds that contain the arylglycerol-β-arylether structure (veratrylglycerol-β-(o-methoxyphenyl) ether and guaiacylglycerol-β-coniferyl alcohol ether), phenylcoumaran compounds (dehydrodiconiferyl alcohol), and biphenyl compounds (dehydrodivanillin) are frequently used to gain knowledge relevant to lignin biodegradation

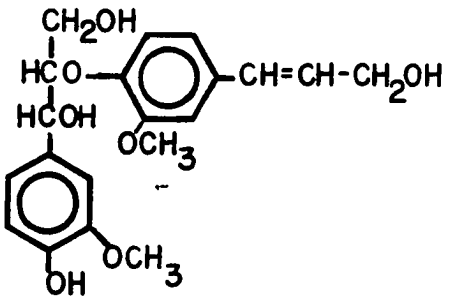
Figure 5. Some representative lignin model compounds. Veratric acid (A), vanillic acid (B), veratrylglycerol-β-(o-methoxyphenyl) ether (C), dehydrodivanillin (D), guaiacylglycerol-β-coniferyl alcohol ether (E), dehydrodiconiferyl alcohol (F), and syringic acid (G).



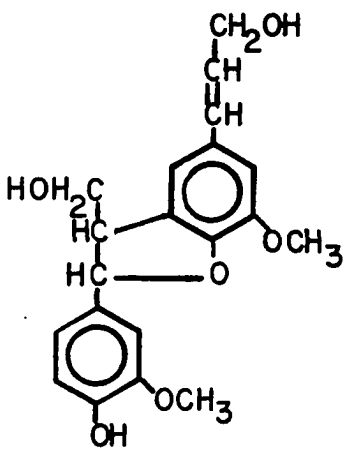
A B C



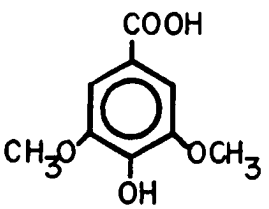
D



E



F



G

(15, 21, 22). Other compounds of varying degrees of complexity have also been utilized as lignin model compounds based upon their structure and intermonomeric linkages (23, 75, 79).

III. Microbial degradation of lignin.

A wide variety of both fungi and bacteria have been shown to degrade lignin. Several reviews of the organisms involved as well as the mechanisms of lignin degradation appear in the literature (15, 18, 48, 58, 80). Due to the complex polymeric structure of lignin, the organisms known to be capable of completely degrading lignin are few in number. Other microorganisms may degrade lignin incompletely because they lack the enzymatic capability to attack all of the varied structural elements of the polymer.

White-rot fungi, mostly Basidiomycetes, have been the most intensively studied of all the wood decay fungi. White-rot fungi simultaneously degrade both the carbohydrate and lignin portions of wood during decay (58). Phanerochaete chrysosporium, Coriolus versicolor, and Pleurotus ostreatus have been shown to degrade ^{14}C-DHPs labeled in the ring, side-chain, or methoxyl groups to $^{14}CO_2$ (29, 43, 44, 62). Recent studies (1, 96) have demonstrated that a variety of Cyathus spp. have the ability to degrade ^{14}C-labeled lignin. The most active, C. pallidus, released about 1% of the total ^{14}C in the sample per day over a 32 day period.

Spruce lignin was shown to be degraded by the rot fungi P. chrysosporium, Polyporous anceps, C. versicolor, and Poria subacida on the basis of chemical analysis of rotted lignins (60, 61). The rotted lignin fragments had a molecular weight greater than 1700, which

corresponds to 8-10 "monomers". Degradation was oxidative with an increase in carbonyl, phenolic hydroxyl, carboxyl, and oxygen/carbon content. Decreases in aliphatic hydroxyl, hydrogen/carbon, and methoxyl/carbon content were also noted. Based upon these findings Kirk et al. (66) have proposed a sequence of catabolic events for lignin degradation by white rot fungi. The fungi first attack the exposed surfaces of the lignin polymer and later decompose more deeply located lignin as it becomes exposed. Demethylation of guaiacyl and syringyl units are the principal early reactions, resulting in the formation of catechol moieties. These are subsequently attacked by fungal dioxygenases in ring fission reactions to produce aliphatic carboxylic acid products which are still bound to the lignin polymer. Further degradation of aliphatic products is proposed to hydrolytic. Also, oxidation of side chains to produce carbonyl groups α to the aromatic rings and aromatic carboxyls may release some low molecular weight lignin fragments from the polymer if β-ether linkages between C-9 units are cleaved. Thus, as erosive decay proceeds, sound, subsurface lignin is exposed and the process begins anew.

Brown-rot fungi comprise numerous species of Basidiomycetes which are similar in their extensive degradation of cellulose and hemicellulose in wood, but only limited degradation of lignin (2, 15, 58). Detailed analysis of sweetgum wood decay by Lenzites trabea has shown that brown- rot attack on lignin is oxidative and that demethoxylation and ring hydroxylation in the 2' position are the major degradative modifications in brown-rotted lignin. These reactions are the same as those observed for ring cleavage pathways for methoxylated single-ring

aromatic compounds such as vanillic and veratric acids (15, 59). Further work by Kirk et al. (62) showed that demethoxylation was the major feature of lignin exposed to the brown-rot fungi Poria coeos and Gloeophyllum trabeum based on the decomposition of specifically labeled DHPs to $^{14}CO_2$. As Kirk (58) points out, the principal difference between the white-rot and brown-rot fungi is believed to be the inability of brown-rotters to cleave aromatic rings or, if cleavage does occur, to metabolize the aromatic products of ring cleavage.

Soft-rot fungi include members of Ascomycetes and fungi imperfecti that have been shown to decompose all major components of wood, including lignin. Strains of Graphium, Monodictys, Paecilomyces, Papulospora, Thielava, and Allescheria were examined by Eslyn et al. (1975) and found to decompose lignin. Preussia, Chaetomium, and Stachybotrys were shown to degrade synthetic lignin in studies by Haider and Trojanowski (43, 44) using DHPs specifically labeled in the ring, side-chain, and methoxyl portions. The chemical nature of soft-rot decayed lignin has yet to be determined.

Several other fungi not classifiable into specific decay groups have also been shown to decompose lignin. A strain of Aspergillus fumigatus degraded 50% of ^{14}C-kraft lignins to $^{14}CO_2$ in 16 days (28). Higuchi (50) and Iwahara (53) have shown that Fusarium strains readily decompose DHPs. Trojanowski et al. (92) examined five different species of ecto-micorrhizal fungi: Cenococcum geophilum, Amanita muscaria, Tricholoma aurantium, Rhizopogon luteolus, and Rhizopogon roseolus. All of these strains were capable of decomposing ^{14}C-labeled plant lignin, ^{14}C-lignocellulose, and ^{14}C-DHP, albeit

at rates lower than those for the white-rot fungi tested simultaneously, Heterobasidion anmosum and Sporotrichum pulveratum (Phanerochaete chrysosporium). Finally, Clayton and Srinivasan (9) demonstrated by gel permeation chromatography that a Candida sp. produced intermediate molecular weight aromatic compounds during growth on a kraft lignin waste.

Numerous reports have appeared in the literature relating to the bacterial degradation of lignin. A number of Norcardia strains have been shown to degrade ^{14}C-DHPs or ^{14}C-[Lignin]-lignocellulose from maize (44, 45, 93). One Norcardia species was shown to decompose 10% of aromatic rings from specifically labeled ^{14}C-DHP to $^{14}CO_2$ over a 15 day incubation period. Values for ^{14}C-[Lignin]-lignocellulose were 15%, 13%, and 5% for methoxyl, side-chain, and ring constituents, respectively (93). Haider et al. (45) screened a number of Norcardia and Pseudomonas species for lignin-degrading capabilities and concluded that strains of N. autotrophica were the most active in decomposing lignin to $^{14}CO_2$. The pseudomonads studied were much less capable of attacking lignin.

Robinson and Crawford (82) have reported on a strain of Bacillus megaterium which released $^{14}CO_2$ from spruce lignin labeled in the side-chains at an initial rate equivalent to that of many lignin-degrading fungi.

Extensive studies on lignin degradation by Streptomyces strains have come from the laboratories of Crawford and Crawford. Streptomyces strains were first demonstrated to convert ^{14}C-milled wood lignin to $^{14}CO_2$ (12). In further studies Phelan et al. (77) examined six

Streptomyces strains for their abilities to decompose lignocellulose and found that both the lignin and glucan components were converted to $^{14}CO_2$ and ^{14}C-water-soluble products. A strain of Streptomyces badius was the most active lignin-decomposing actinomycete examined, degrading 13% of ^{14}C-[Lignin]-lignocellulose to $^{14}CO_2$ in 1008 hours at 35°C. Chemical analysis of lignin degraded by S. viridosporus indicated that decomposition is oxidative and involves demethylation, ring cleavage reactions, and attack on phenylpropanoid side-chains. Also, some low molecular weight intermediates were released from the polymer (13). Further studies with this organism (17) revealed the release of an acid-precipitable polyphenolic, polymeric lignin (APPL) intermediate during lignin degradation. The APPL was a water-soluble polymer of a heterogenous mixture of molecular weight components $\geq$20,000. The authors proposed APPL production by oxidative cleavage of p-hydroxyether linkages and methoxyl groups in the lignin. Recent comparison of APPLs produced by S. viridosporus and S. badius have shown that both are released as the result of β-ether cleavage and other oxidative reactions (6). However, after release as a water-soluble catabolite, low molecular weight intermediates of S. badius degradation of lignin are repolymerized with APPLs in free radical coupling reactions catalyzed by an extracellular phenol oxidase resulting in a higher average molecular weight product.

Recently, Kerr et al. (56) isolated a strain of Arthrobacter capable of degrading peanut hull lignin, ^{14}C-[Lignin]-lignocellulose and ^{14}C-kraft lignin. Odier and Monties (73) demonstrated that strains of Flavobacterium, Pseudomonas, and Aeromonas were capable of

degrading acidolysis lignin, and a strain of Xanthomonas was reported to degrade 77% of the dioxane lignin provided in a medium as a sole carbon source.

More recently Chon (8) has isolated an Erwinia sp. from sewage and demonstrated growth on lignin as a sole carbon source, degradation of the lignin model compounds syringaldehyde and vanillin, and decomposition of kraft lignin to lower molecular weight aromatic compounds.

The anaerobic decomposition of lignin has received much less attention. Early studies (97) failed to demonstrate the mineralization of ^{14}C-DHPs or ^{14}C-labeled natural lignin to $^{14}CO_2$ or $^{14}CH_4$ by mixed anaerobic cultures. However, more recently Benner et al. (3) reported that both synthetic and natural lignins were degraded slowly to gaseous end products by microflora of anoxic sediments. Also, studies by Colberg and Young (10, 11) have shown that soluble oligolignols prepared by alkaline heat treatment of ^{14}C-[Lignin]-lignocellulose are degraded anaerobically to $^{14}CO_2$ and $^{14}CH_4$. In addition, up to 30% of the entire soluble lignin-derived carbon was mineralized to gaseous end products in these studies. Thus, it appears that anaerobic microbial consortia have the capacity for the decomposition of. lignin.

IV. Culture parameters which affect lignin degradation.

Most of the physiological and biochemical investigations of the physiology of lignin degradation have been conducted with Phanerochaete chrysosporium and a number of basic parameters have been optimized (65). Culture agitation results in mycelial pellet formation and suppression

of ligninolysis. The optimum pH for lignin degradation with P. chrysosporium was found to be 4-4.5, with sodium 2-2'dimethylsuccinate serving as an ideal buffer. Furthermore, thiamine is required for growth and the balance of trace elements is important.

Expression of ligninolytic activity in response to nitrogen starvation has been substantiated in a number of studies (33, 57, 65, 95). The source of nitrogen (NO_3^-, NH_4^+, amino acids) had little influence on lignin decomposition, but high nitrogen concentration (24 mm) in the medium decreased ligninolytic activity to levels 23-35% of that at 2.4 mm nitrogen (65).

Oxygen partial pressure also affects lignin metabolism by P. chrysosporium, but not the growth of the organism (65). In a nitrogen-limited medium there was essentially no attack on the lignin polymer when the O_2 concentration in the gas phase above the non-agitated culture was 5% O_2 in N_2. One hundred per cent O_2 resulted in a 2-3 fold enhancement of lignin decomposition as opposed to air (21% O_2).

The lignin degrading system in P. chrysosporium is not inducible by lignin and lignin does not serve as a growth substrate (57, 65). A growth substrate such as cellulose, glucose, xylose, glycerol, or succinate is required for the degradation of ^{14}C-DHPs to $^{14}CO_2$, with the amount of $^{14}CO_2$ released from ^{14}C-DHPs being dependent on the amount of cosubstrate provided.

The effect of sulfur on lignin degradation by P. chrysosporium has also been studied (55). Lignin degradation was shown to be derepressed in 7 days on depletion in cultures limited for $SO_4^=$ (20 mM) that also contained nonlimiting levels of carbohydrate and nitrogen.

In summary, it is apparent that the ligninolytic system of P. chrysosporium is synthesized as a secondary metabolic event in response to the limitation of N, C or S. Furthermore, ligninolytic activity is manifested irrespective of the presence of lignin in the culture medium, and lignin fails to induce enhanced activity.

V. Involvement of reduced oxygen species in the biological decomposition of lignin.

Since lignin biodegradation is an extracellular, oxidative, and relatively nonspecific process it has been speculated that the actual extracellular agent attacking the lignin polymer might be a highly reactive oxygen species or an extracellular enzyme.

Hall (46) suggested that lignin degradation might be mediated by "activated oxygen", i.e. reduced oxygen species such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), or singlet oxygen ($^1O_2$). As a result, many laboratories initiated investigations of the "activated oxygen hypothesis" of lignin decay. Reviews of these studies now appear in the literature (20, 80).

The suggestion that $^{\cdot}OH$ may be the extracellular agent involved in lignin degradation by Phanerchaete chrysosporium was furthered by Forney et al. (35) based on the facts that: (i) $^{\cdot}OH$ is highly reactive and oxidizes various organic compounds and (ii) $^{\cdot}OH$ is nonspecific and can be produced by one electron reduction of H_2O_2, which is generated in substantial amounts by a number of white-rot fungi (67). Evidence that H_2O_2-derived $^{\cdot}OH$ has a role in the ligninolytic system of P. chrysosporium includes the following findings. (i) There was a temporal correlation between H_2O_2 production, $^{\cdot}OH$ production, and

ligninolytic activity. (ii) $^{\bullet}OH$ was produced by ligninolytic cultures as shown by ethylene production from α-keto-γ-methiolbutyric acid, hydroxylation of p-hydroxybenzoic acid to form protocatechuic acid, and electron spin resonance data. (iii) Growth in a low nitrogen medium (2.4 mM) resulted in a concomitant production of $^{\bullet}OH$ and increased ligninolytic activity. (iv) Lignin degradation was suppressed in the presence of $^{\bullet}OH$-scavenging agents (e.g. benzoate). Similar findings have been reported by Kutsuki and Gold (68).

Faison and Kirk (30) have demonstrated a correlation between the kinetics of synthesis of H_2O_2 and the appearance of the ligninolytic system in P. chrysosporium. Also, they observed a marked enhancement of H_2O_2 production by growth under 100% O_2, mimicking the increase in ligninolytic activity seen with cultures grown under elevated oxygen tension. Furthermore, addition of the H_2O_2 scavenger, catalase, resulted in an inhibition of lignin degradation by whole cultures of P. chrysosporium, implying a role for H_2O_2 in the degradation process. Finally, glucose oxidase appears to be the main enzyme involved in the production of H_2O_2, which may ultimately be the source of $^{\bullet}OH$ in ligninolytic cultures of P. chrysosporium (80).

Faison and Kirk (31) also noted that superoxide dismutase also inhibited lignin degradation, suggesting a role for $O_2^{\bullet}$ in lignin breakdown. However, as Reddy (80) points out, $O_2^{\bullet}$ is relatively nonreactive in aqueous systems and may be involved in generating $^{\bullet}OH$ in ligninolytic systems by the iron-catalyzed Haber Weiss reaction:

$$\text{Fe(III) chelate} + O_2^{\bullet} \rightarrow \text{Fe(II) chelate} + O_2$$

$$\underline{\text{Fe(II) chelate} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + \text{Fe(III) chelate}}$$

$$\text{Sum: } H_2O_2 + O_2^{\bullet} \rightarrow {}^{\bullet}OH + O_2.$$

When similar investigations using singlet oxygen and hydroxyl radical scavengers were carried out with the ligninolytic bacterium Streptomyces viridosporus (20), it was concluded that S. viridosporus does not generate significant extracellular activated oxygen species as a component of its ligninolytic system. Such findings suggest that different ligninolytic organisms may differ also in their mechanism of attack on the lignin polymer.

VI. Enzymes involved in lignin biodegradation.

Until 1983 there had been no enzymes described in any microbial system that were shown to catalyse any of the numerous complex reactions known to occur during lignin biodegradation. It was then that Tien and Kirk (90, 91) described an enzyme from the supernatants of ligninolytic cultures of P. chrysosporium which degrades lignin model compounds as well as spruce and birch lignins. The enzyme was purified to homogeneity by ion-exchange chromatography and shown to have a molecular weight of 42,000 by SDS-PAGE. It exhibits both peroxidase and oxygenase activities, contains one Fe atom per molecule, and has maximum activity at pH 3.0. The enzyme nonspecifically catalyzes several oxidations in the alkyl side-chains of lignin-related compounds: C^{α}-C^{β} cleavage of β-1 diarylpropane, β-1 diarylethane, and β-O-4 ether dimers, oxidation of benzyl alcohols to aldehydes or ketones, intradiol cleavage in phenylglycol structures, and hydroxylation of benzylic methylene groups. All reactions require H_2O_2.

Recent studies (32) show that the gross regulation of the "ligninase" activity correlates with that of the complete ligninolytic system. Both activities are idiophasic and appear in cultures limited

for carbon or nitrogen and are suppressed by supplementation with these nutrients. Increased activities of both are seen under 100% O_2. Further, it appears that this enzyme is inducible by lignin and its degradation products, in contrast to the entire ligninolytic system, which has been considered noninducible by lignin (57, 65).

Glenn, et al. (39) described an extracellular enzyme from ligninolytic cultures of P. chrysosporium that seems to be similar, if not the same as, the enzyme of Tien and Kirk (90, 91). This enzyme also requires H_2O_2, generates ethylene from KTBA, acts as a nonspecific oxygenase, and partially degrades ^{14}C-ring labeled lignin to lower molecular weight material. Studies with model compounds show the enzyme catalyzes Cα-Cβ bond cleavages, and hydroxylation and subsequent cleavage of an aromatic olefin.

In a unique report (20) a biomimetic approach using a model catalyst was used to study the mechanism of lignin degradation and the generation of the active oxygen species involved. It was shown that tetraphenyl- porphyrinatoiron (III) chloride catalyzed oxidative cleavage of the Cα-Cβ bond of a β-1 diarylpropanediol model compound. It was hypothesized that the actual active oxygen species may be in a form of a high-valent oxo-metalloporphyrin complex.

Another enzyme found in ligninolytic cultures of P. chrysosporium has been described (20) which demethylates lignin model polymers such as polyguaiacol. Apparently the methyl moiety is released as methanol or formaldehyde rather than CO_2 or CO. This enzyme also requires H_2O_2 for activity.

These studies substantiate the role of an activated oxygen species in the ligninolytic system of P. chrysosporium, and also suggest that the oxygen may be held in the active site of an extracellular enzyme.

Work by D.L. Crawford and colleagues (20) has shown that Streptomyces viridosporus produces an enzyme system involved in the cleavage of β-ether linkages in lignin. This system appears to be inducible by lignin or APPLs. However, ether cleavage is not stimulated by H_2O_2. Studies with the β-O-4 model compound, veratrylglycerol-β-guaiacol ether have shown that this compound is initially demethylated to form the guaiacylglycerol-β-guaiacyl ether. Then, the α-hydroxyl group of the propane side-chain is oxidized to the α-carbonyl. Upon side-chain oxidation, cleavage occurs, with guaiacol as one product. The phenylpropane cleavage product, which is transitory, is rapidly degraded through vanillin and vanillic acid. Thus, it has been hypothesized that this enzyme complex is involved in producing APPLs as intermediate products in lignin degradation by S. viridosporus.

VII. Potential applications of bioligninolytic systems.

Presently, there is no industrial process for the bioconversion of lignin into useful products (24). However, there is great potential for the development of such processes which might produce a variety of chemicals now derived from petroleum. The increasing costs of oil-based chemicals will eventually necessitate the utilization of a renewable raw material. Lignin is a prime candidate because of its polyphenolic structure and abundance.

The sources of lignin-containing materials can be divided into three main categories: native lignocellulose, pulp process liquors, and solid waste (24). Wood, as an example of native lignocellulose, is a good candidate for bioconversion because large quantities could be made available for processing. Current tree-harvesting programs leave 20-50% of the total biomass in the forest (52). With about 240 million tons of wood per year being harvested, wood residues are substantial in quantity.

In the pulp and paper industry, the kraft pulping process accounts for 98% of the chemical pulp production in the United States (24). Kraft lignin production amounts to approximately 16 million tons per year. At present, only limited uses have been found for kraft lignins. Most are burned in chemical recovery processes (52). Kraft lignins are also utilized as additives in products such as emulsifiers, stabilizers, grinding aids for cement, dispersants for clays and ceramics, binders and adhesives for roads, and oil well drilling muds (52). Kraft lignin has been shown to be degraded by both fungi (34) and bacteria (16).

Solid waste represents a third major source of lignin. It has been estimated that about 100 million tons of urban and municipal wastes and cattle manure are generated per year in the United States. Of this about 50% is lignocellulose (24).

One potential application of bioligninolytic systems is biological pulping by preferential removal of lignin from wood. It has been pointed out recently that even limited fungal removal of lignin from wood chips (3-4%) in the absence of cellulose degradation considerably reduced the energy requirements for additional mechanical pulping of the wood (15).

There is also great potential for the conversion of lignin to low molecular weight chemicals. At present a limited number of chemicals are produced from lignin by degradative chemical processes (41). Low molecular weight chemicals such as vanillin, dimethyl sulfide, dimethyl sulfoxide, and methyl mercaptan are produced in low yields by controlled chemical hydrolysis of waste lignin sulfonates or kraft lignin under high temperature and pressure. Phenols can also be produced from lignin by high temperature pyrolysis, but not at economical yields. Such processes are highly energy intensive. Application of microbial lignin conversion processes, once developed, should lessen energy requirements. Utilization of specific microorganisms (or enzymes) which alter lignin in specific ways may allow for production of useful degradation products.

It is clear from the literature reviewed here that selected microbial strains will become utilized in studies directed at lignin bioconversion. The microorganisms involved may be genetically manipulated for the overproduction of useful degradation products. Further catabolism of aromatic lignin intermediates may necessitate genetic blockage of steps in lignin metabolism, such as aromatic ring cleavage, by classical mutagenesis techniques. Alternatively, gene cloning into a suitable host may also allow expression of select enzymatic activities.

Most microbial degradation of lignin studied thus far requires long periods of time, e.g. weeks and months, to achieve even limited decomposition. Again, genetic manipulation for the overproduction of ligininolytic activities to speed bioconversion processes may be a

useful approach. Protoplast fusion has been used by Pettey and Crawford (77) in this regard to construct APPL-overproducing strains of Streptomyces. Gene cloning into appropriate multicopy plasmids and expression vectors containing strong promoters also appears promising in the engineering of bioligninolytic processes.

Upon review of the literature one acquires an appreciation for the recalcitrance of lignin to microbial degradation. A variety of microorganisms examined thus far have demonstrated at least a limited ability to decompose lignin. However, the heterogeneous structure of the polymer, maintained by numerous types of intermonomeric bonds, results in a high degree of resistance to microbial attack. As a result microbial degradation of lignin is very slow, and for this reason development of bioligninolytic processes has not yet been realized.

Some progress has been made in characterizing the oxidative extracellular enyzmes of Phanerochaete chrysosporium. Future research with these and other microbial enzymes involved in lignin catabolism may eventually lead to economically feasible bioprocessing of lignin.

MATERIALS AND METHODS

I. Bacterial strains.

The *Erwinia* sp. used in these studies was isolated and identified by Y. Chon (8).

Escherichia coli strains and genotypes were: i) HB101 (F-hsdS20, [r_β^-,m_β^-], recA13, ara-14, proA2, lacY1, galK2, rpsL20[sm^r], xyl-5, mtl-1, supE44, λ^-). ii) Cs412 (HsdR, pro, leu, strA, lacY1, thi-1, thr-1, tonA21, supE44, λ^-). iii) RC30 (lacI22, lac258, relA1, spoT1, thi-1,λ^-). This strain was obtained from B. Bachman, *E. coli* Genetic Stoc Center, Yale School of Medicine, iv) NM538 (supF, hsdR derivative of ED8654) (38), v) NM539 (supF, hsdR, [P2cox3]) (38), vi) BHB2688 (N205 $recA^-$, [λ^- imm^{434}, cIts, b2, red^-, Eam, Sam]/λ) (51), vii) BHB2690 (N205 $recA^-$, [λ imm^{434}, cIts, b2, red^-, Dam, Sam]/λ) (51), and viii) JM103 ([lac, pro], thi strA, SupE, endA, sbcB, hsdR, F'traD36, proAB, lacI , lacZ Δ m15) (71).

These bacterial strains are available from the Department of Microbiology Culture Collection, Louisiana State University.

II. Media.

The *Erwinia* sp. was grown on a minimal medium of the following composition (per liter): 5 g glucose, 1.5 g $(NH_4)_2SO_4$, 0.36 g K_2HPO_4, 0.12 g $NaH_2PO_4 \cdot H_2O$, 0.01 g $CaCl_2$, 0.1 g $MgCl_2 \cdot 6H_2O$, 2.5 x 10^{-3}g $FeSO_4 \cdot 7H_2O$, 8 x 10^{-4}g $ZnSO_4$, 2 x 10^{-5}g $CoCl_2$, 5 x 10^{-7}g $CuSO_4 \cdot 5H_2O$, and 1 x 10^{-5}g $MnCl_2 \cdot 4H_2O$. The pH was adjusted to 7.0 with 0.2 N NaOH.

The *E. coli* strains were grown on the following media (per liter): i) LB: 10 g Bacto-tryptone (Difco), 5 g Bacto-yeast extract (Difco), and 5 g NaCl. The final pH was 7.4; ii) M9 minimal medium: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl. After sterilization and

cooling the following constituents were added: MgSO to 2 mM, glucose to 0.2%, and CaCl to 0.1 mM. Amino acids and vitamins were added as required. The final pH was 7.4; iii) NZC: 10 g NZ amine (Sheffield Products), 5 g NaCl, 1 g casamino acids (Difco), and 2 g $MgSO_4 \cdot 7H_2O$. The final pH was 7.4; iv) SOB: 10 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.4 g KCl, 2 g $MgCl_2$, and 2.5 g $MgSO_4$. Magnesium salts were added as filter sterilized solutions after autoclaving. The final pH was 7.2. v) SOC: SOB medium supplemented with 3.6 g glucose per liter and vi) MacConkey agar was purchased from Difco.

For solid media, 15 g of Bacto-agar (Difco) was added per liter.

Ampicillin was added to a final concentration of 40 µg/ml when necessary; tetracycline was used at 15 µg/ml.

III. Vectors.

The plasmid vectors used in these studies were pBR322 (5) and pCQV2 (78). The phage vectors utilized were the lambda replacement vector EMBL4 (38), M13mp18, and M13mp19 (71). λgt4 lac5 was obtained from S. Chang, Louisiana State University. These vectors are available from the Department of Microbiology Culture Collection, Louisiana State University.

IV. Enzymes used in molecular cloning.

Restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase, DNA polymerase I and the large fragment of DNA pol I (Klenow) were purchased from BRL and used according to the supplier's recommendations.

V. Methods of DNA preparation.

a. Erwinia sp. chromosomal DNA. Cells were grown overnight in one liter of the minimal salts medium plus glucose at 37°C with shaking (200

rpm). The cells were then sedimented by centrifugation at 5000 x g in a Sorvall GSA rotor for 10 minutes at 4°C. The cellular wet weight was estimated and the cells were resuspended in a solution of 0.1 M EDTA, pH 8.0, containing 0.15 M NaCl and 2 mg/ml lysozyme (Sigma). One ml of solution was added per gram of wet weight. The suspension was then incubated at 37°C without shaking for 20 minutes. Next, 10 ml per gram of wet weight of a solution of 0.1 M Tris·Cl, pH 8.0, containing 0.1 M NaCl and 1% SDS were added. Two cycles of freezing the cellular suspension at -20°C and thawing at 65°C were performed. An equal volume of redistilled phenol saturated with 50 mM Tris·Cl, pH 8.0, containing 50 mM NaCl and 5 mM EDTA was added and the emulsion was incubated at 4°C for 20 minutes with occasional shaking. The emulsion was then centrifuged at 12,000 rpm (Sorvall GSA rotor) for 20 minutes at 4°C and the aqueous layer was pipetted into sterile centrifuge bottles. The DNA was precipitated by the addition of 2 volumes of cold ethanol and sedimented by centrifugation at 12,000 rpm (GSA rotor) for 20 minutes at 4°C. The DNA was resuspended in 2 ml of 10 mM Tris Cl, pH 8.0, containing 1 mM EDTA (T.E.) and 40 µl RNase (10 mg/ml) was added, followed by incubation at 37°C for 30 minutes. Then, pronase was added to a final concentration of 500 µg/ml and incubation at 37°C was continued for 30 minutes. The solution was then extracted once with an equal volume of T.E. saturated phenol, once with phenol: chloroform (1:1), and three times with water-saturated ether. Residual ether was removed by heating at 65°C for 5-10 minutes with occasional shaking until none could be detected. After cooling to room temperature, 1/10 volume of 3 M sodium acetate, pH 4.8, was added and the DNA was

precipitated with 2 volumes of ethanol at -20°C for > 2 hours. The DNA was sedimented by centrifugation at 12,000 rpm (GSA rotor) for 15 minutes at 4°C, washed once with 80% ethanol, and dried in vacuo. Finally, the DNA was resuspended in 1.5 ml T.E. and the concentration was estimated by measuring the absorbance at 260 nm.

b. Plasmid DNA. Five ml of an overnight culture of cells grown in LB plus 40 µg/ml ampicillin were used to inoculate 1 liter of either LB or M9 media containing 40 µg/ml ampicillin. (It should be noted that cells grown on minimal medium lysed more readily in the following procedure.) The culture was grown at 37°C (32°C in the case of pCQV2) with shaking (250 rpm) to a cell density of 100 Klett units. Plasmid DNA was amplified by adding chloramphenicol (150 mg/l) and continuing incubation overnight with shaking. The cells were sedimented by centrifugation for 5 minutes at 5,000 x g to yield a soft pellet. The cells were then resuspended in 5 ml of a solution of 50 mM Tris·Cl, pH 8.0, containing 25% sucrose. Lysozyme (0.5 ml of a solution of 50 mM Tris·Cl, pH 8.0, containing 10 mg/ml lysozyme and 25% sucrose) was added and the suspension was incubated on ice for 5 minutes. Then, 0.5 ml of 0.25 M EDTA, pH 8.0, was added and incubation was continued for 5 minutes. Next, 5 ml of Triton X-100 lytic mixture (50 mM Tris·Cl, pH 8.0, containing 62 mM EDTA and 0.4% Triton X-100) was added quickly, vortexed briefly, and left at room temperature for 5 minutes or until a clear lysate was achieved. Cellular debris was sedimented by ultra-centrifugation at 40,000 rpm for 45 minutes in a Beckman type 75Ti rotor. The supernatant fluid was decanted into a plastic centrifuge tube. One gram of CsCl and 0.1 ml of a solution of 5 mg/ml ethidium

bromide were added per ml of supernatant. Protein debris was then removed by centrifugation at 10,000 rpm (Sorvall SS34 rotor) for 5 minutes. The supernatant was transferred to plastic quick-seal tubes and the gradients were centrifuged to equilibrium at 49,000 rpm in a Beckman Vti65 rotor for 20 hours at 15°C. Covalently closed circular plasmid DNA was visualized with long-wave ultraviolet light and plasmid bands were withdrawn from the side of the tubes with a number 21 needle and syringe. To remove th ethidium bromide, the plasmid DNA in CsCl solution was extracted 6 times with water-saturated butanol. The solution was then diluted with two volumes of water. DNA was precipitated by the addition of 6 times the original volume of ethanol and freezing at -20°C for >2 hours. The DNA was pelleted by centrifugation at 10,000 x g, washed with 80% ethanol, dried in vacuo, and resuspended in T.E. buffer.

c. Small-scale plasmid isolation. Small quantities of plasmid DNA were prepared for rapid screening by the method of Birnboim and Doly (4) with some modifications. Cell containing plasmids were grown overnight in 5 ml of the appropriate medium. One and one-half ml of culture was transferred to a microfuge tube and centrifuged for 30 seconds to pellet the cells. The supernatant was removed and the cells were resuspended in 110 µl of a solution of 25 mM Tris·Cl, pH 8.0, containing 5 mM glucose, 10 mM EDTA, and 2 mg/ml lysozyme. The suspension was incubated for 30 minutes on ice. Then, 220 µl of a solution of 0.2 N NaOH containing 1% SDS was added and mixed by inversion until a clear lysate was achieved. The lysate was kept on ice for 5 minutes before the addition of 165 µl of 3 M sodium acetate, pH 4.8. The tubes were then

placed on ice for 60 minutes, during which time a clot of DNA and cellular debris formed. The debris was pelleted by centrifugation for 5 minutes. The supernatant (400 µl) was then transferred to a second microfuge tube, 1 ml of ethanol was added, and plasmid DNA was precipitated at -70°C for 30 minutes. The DNA was sedimented by centrifugation for 10 minutes and the supernatant was discarded. The DNA was resuspended in 100 µl of 0.05 M Tris·Cl, pH 8.0, containing 0.1 M sodium acetate and then reprecipitated with 2 volumes of ethanol at -70°C for 10 minutes. After sedimenting by centrifugation, the DNA was dissolved and reprecipitated a third time. The DNA pellet was washed with 80% ethanol, dried in vacuo, and resuspended in 40 µl T.E. buffer.

d. Preparation of lambda vector DNA. Host cells (NM538) were grown overnight in NZC medium supplemented with 0.2% maltose. The cells were then centrifuged at 7,500 rpm for 5 minutes at room temperature and resuspended in 1/10 volume SM (50 mM Tris Cl, pH 7.5, containing 100 mM NaCl, 10 mM $MgSO_4$, and 0.01% gelatin) to give a final cell density of about 10^{10} per ml. EMBL4 (5×10^7 p.f.u.) was then added to aliquots containing 10^{10} cells and allowed to absorb by incubating at 37°C for 20 minutes with intermittent shaking. Each infected aliquot was then added to 100 ml of prewarmed NZC broth in side-arm flasks and incubated at 37°C with vigorous shaking (300 rpm). Optical density was measured at hourly intervals to follow growth and lysis of the cultures, which usually occurred in 4 to 5 hours. The lysed cultures were pooled in a sterile flask and DNase I and RNase were added to 1 µg/ml, followed by incubation at room temperature for 30 minutes. Solid NaCl was then

added to a final concentration of 1.0 M, swirled to dissolve, and placed on ice for 1 hour. Cellular debris was then removed by centrifugation at 11,000 x g for 10 minutes at 4°C. The supernatant was then transferred to a sterile flask and PEG 6000 was added to 10% (w/v). The PEG was dissolved by stirring and placed at 4°C overnight to precipitate the phage. Phage particles were sedimented by centrifugation at 21,000 x g for 20 minutes. The phage pellet was resuspended in 5.3 ml SM and an equal volume of chloroform was added and rocked gently to mix. The resulting emulsion was broken by centrifugation at 1,600 x g for 15 minutes at 4°C. The aqueous phase was removed and its volume measured. An equal volume of 65% CsCl (w/w) was added and the mixture was placed in nitrocellulose tubes. The gradients were centrifuged to equilibrium at 40,000 rpm in a Beckman SW65 rotor for 24 hours at 15°C. The bluish-gray band of phage was removed from the side of the tube with a number 21 needle and syringe. The phage suspension was dialysed twice for 1 hour against 500 ml of 50 mM Tris·Cl, pH 8.0, containing 10 mM NaCl and 10 mM $MgCl_2$. Pronase was then added to 1 mg/ml and the suspension was incubated at 37°C for 30 minutes to remove the protein coat. Next, an equal volume of T.E.-saturated phenol was added to extract proteinaceous material. The emulsion was broken by centrifugation at 10,000 rpm (SS34 rotor) for 10 minutes at 4°C. The aqueous phase was removed and the organic volume of T.E. buffer. The aqueous phases were combined, 1/10 volume of 3 M sodium acetate, pH 4.8, was added, and phage DNA was precipitated with 2 volumes of ethanol at -20°C for $>$ 2 hours. The DNA was sedimented by centrifugation at 10,000 x g for 20 minutes at 4°C.

The pellet was washed with 80% ethanol and dried in vacuo. Finally, the phage DNA was resuspended in T.E. buffer and the concentration was estimated by measuring the absorbance at 260 nm.

e. Purification of M13 RF DNA. To prepare a phage lysate, host cells (JM103) were grown overnight in 5 ml of LB broth and then diluted 1:50 in fresh LB broth. A single plaque of either M13mp18 or M13mp19 was plugged with a sterile pasteur pipet and used to infect 2 ml of freshly diluted host cells. The cultures were grown overnight at 37°C with shaking (250 rpm). The cells were then sedimented by centrifugation in a microfuge. The supernatant was transfered to a sterile microfuge tube. Host cells were then grown to A_{600} = 0.6 and diluted 1:50 into 150 ml LB broth. The culture was infected with 50 µl of the phage lysate and grown at 37°C with shaking (250 rpm) for 7 hours. The cells were harvested by centrifugation at 7,000 rpm (SS34 rotor) for 10 minutes at 4°C. Cells obtained in this manner were then lysed by a scaled-up version of the rapid plasmid isolation technique of Birnboim and Doly (4) described previously. Replicative form (RF) DNA was purified by CsCl, ethidium bromide density gradient centrifugation as described previously.

f. Preparation of M13 single-stranded template DNA. An overnight culture of JM103 was diluted 1:40 in fresh LB broth and grown to an $O.D._{650}$ = 0.3 at 37°C with shaking (250 rpm). Aliquots (1 ml) were transferred into the culture with a sterile pasteur pipet. The cultures were incubated with shaking (200 rpm) at 37°C for 4½ hours and then transferred to microfuge tubes. The tubes were centrifuged for 5 minutes and the supernatant was transferred to another microfuge tube.

Next, 200 µl of 20% PEG 6000 in 2.5 M NaCl was added, mixed, and left at room temperature for 30 minutes. Phage were then pelleted by centrifugation for 10 minutes and the supernatant was discarded. The pellet was then resuspended in 100 1 T.E. buffer. The phage were uncoated by extraction with an equal volume of phenol over a 10 minute period. After centrifugation, the aqueous layer was transferred to another tube, 3 M sodium acetate was added to 0.3 M, and the DNA was precipitated by the addition of 2 volumes of ethanol and incubation at -70°C for ten minutes. The templates were then pelleted by centrifugation for 10 minutes, washed once with 80% ethanol, dried in vacuo, and resuspended in T.E. buffer.

VI. In vitro packaging of recombinant lambda DNA.

Packaging extracts were prepared essentially by the protocol described in Maniatis, et al. (69) with slight modification. The E. coli lysogens BHB2690 (Dam) and BHB 2688 (Eam) were used in this procedure.

Each strain was grown overnight in 50 ml of M9 medium containing 0.2% casamino acids at 32°C with shaking (250 rpm). Each culture was then diluted into 500 ml of M9 medium to give an initial $O.D._{600}$ of about 0.1. The cells were grown with vigorous shaking (300 rpm) until the $O.D._{600}$ was 0.3. The lysogens were then induced by placing the flasks in a 45°C water bath and incubating for 15 minutes after the cultures had reached 45°C. The induced cells were then incubated at 39°C for 3 hours with vigorous shaking. Successful induction was noted by the clearing of a 2 ml sample of the culture upon the addition of a drop of chloroform. The two lysogens were then mixed and chilled in an

ice-water bath before recovering cells by centrifugation at 4,000 x g for 10 minutes at 4°C. The cells were then washed in 300 ml of ice-cold M9 medium without casamino acids and recovered by centrifugation as above. The supernatant was discarded and the centrifuge bottles were inverted on paper towels in a cold room to drain away excess liquid from the cell pellet. Remaining liquid was removed with a pasteur pipet and the walls of the centrifuge bottles were dried with Kimwipes. A siliconized pasteur pipet was used to resuspend the cell pellet in 4 ml of CH buffer (40 mM Tris·Cl, pH 8.0, containing 10 mM spermidine, 10 mM putrescine, 0.1% 2-mercaptoethanol, and 7% DMSO). The cell suspension was then transfered to a small tube and 50 µl aliquots were dispensed into microfuge tubes using wide-bore pipet tips. Each tube was plunged into liquid nitrogen and the entire lot was stored at -70°C.

DNA (0.1-1.0 µg) to be packaged was prepared in 5 µl of 66 mM Tris·Cl, pH 8.0, containing 10 mM $MgCl_2$ or was used immediately after ligation in 50 mM Tris·Cl, pH 7.4, containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP, and 1 mg/ml BSA. The solution of DNA was mixed with 10 µl of CH buffer and 1 µl of 0.1 M ATP, pH 7.5. (The amount of CH buffer to be used was determined by carrying out in vitro packaging of 0.5 µg intact EMBL4 DNA using 0-50 µl CH buffer. The highest titers, about 10^6 pfu/µg of EMBL4 DNA were obtained using 10 µl CH buffer in the above mixture.) This mixture was then added to 50 µl of the frozen packaging extracts and blended with a sealed capillary pipet as the extracts thawed. After mixing well, the reaction was carried out at 37°C for 60 minutes. To a second packaging extract, 5 µl of pancreatic DNase (10 µg/ml) and 2.5 µl of 0.5 M $MgCl_2$ were added as

the extracts thawed. The mixture was stored on ice for 10 minutes before adding 20 µl to the original packaging reaction. Incubation was continued at 37°C for 30 minutes after addition of the second extract. Finally, 1 ml of SM and 5 µl of chloroform were added to the packaging reaction.

The number of viable phage particles was estimated as follows. An overnight culture of the appropriate host (NM 538 or NM 539) was diluted 1:50 in LB medium plus 0.2% maltose and grown at 37°C to an $O.D._{600}$ of 0.6. The cells were then sedimented at 4,000 x g for 10 minutes at 4°C. The cell pellet was resuspended in SM to give an $O.D._{600} = 2$. Ten-fold serial dilutions of the phage were prepared in SM and 0.1 ml of each appropriate dilution was added to 0.1 ml host cells. After mixing, the suspension was incubated at 37°C for 20 minutes to allow adsorption of the phage. Then, 3.0 ml of LB medium containing 0.7% agar (LB top agar) was added to each suspension and then poured onto an LB plate. The plates were incubated at 37°C before scoring the plaques.

VII. Transformation of E. coli.

Competent cells of the appropriate host were prepared and transformed with either plasmid or M13 RF DNA by the method of Hanahan (47).

VIII. Thin layer chromatography (TLC).

TLC analysis of lignin and model compound transformation was performed by Y. Chon as described (8). Briefly, cell pellets of Cs412 harboring pNC1 or pNC2 from 10 ml cultures grown in M9 medium with 0.5% salicin as the carbon source were resuspended in 1 ml of 10 mM Tris·Cl, pH 7.8, containing 10 mM $MgCl_2$ and 0.1% lignin or model compound. After

incubation, supernatants were analyzed by TLC. Solvent systems were: i) Benzene, 2-propanol, NH_4OH (4:1:1) and ii) Benzene, propionic acid, water (2:2:1).

IX. Qualitative PNPG assays.

Broth cultures (1.5 ml) were pelleted in microfuge tubes and resuspended in 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.5. p-Nitrophenyl-β-D-glucopyranoside (PNPG, Sigma) (100 µl) was added and the tubes were incubated at 37°C until the appearance of a yellow color.

X. Electrophoretic techniques.

Horizontal agarose gels were prepared in T.B.E. electrophoresis buffer (89 mM Tris-borate, pH 8.0, containing 2 mM EDTA). Loading buffer (6X) contained 0.25% bromphenol blue, 0.25% xylene cyanol, and 15% Ficoll in H_2O. After running, gels were stained in a solution of ethidium bromide (0.5 mg/ml) for 30-60 minutes. DNA was visualized on a U.V. transilluminator. Drigest molecular weight markers were purchased from P.L. Biochemicals.

DNA was recoved from agarose gels by one of the following methods. When DNA was plentiful, the desired DNA was excised in a slice of the agarose gel. The gel was then macerated in 5 ml of 5X T.B.E. buffer and incubated overnight at 37°C to allow diffusion of the DNA out of the gel bits. Pieces of the gel were then removed by passing the solution through a syringe plugged with siliconized glass wool. The DNA was then further concentrated and purified using an Elutip-D column (Schleicher and Schuell).

When small quantities of DNA were available, a slot was cut in the gel just ahead of the desired DNA. A piece of dialysis membrane was

used to line the bottom and distal side of the slot. The slot was filled with T.B.E. buffer and electrophoresis was continued until the DNA had run against the dialysis mambrane. The current was reversed for 1-2 minutes to pull the DNA off of the membrane. The buffer containing the DNA was then withdrawn and the DNA was purified with an Elutip-D column.

For DNA sequencing, 6.7% or 6.7% buffer gradient (0.5-2.5 x T.B.E.) polyacrylamide gels containing 8 M urea were used to fractionate ^{35}S-labeled DNA transcripts. Loading buffer was 0.1% bromphenol blue, 0.1% xylene cyanol, and 10 mM EDTA in deionized formamide. Sequencing gels were run at 25 watts for 5 hours (6% gels) or for 2.5 hours (gradient gels). T.B.E. (0.5x) was used as the running buffer in the upper reservoir and 1 x T.B.E. was used in the lower reservoir. Gels were fixed in a solution of 10% methanol and 10% glacial acetic acid in water before transfer to 3 mm paper and drying at 80°C on a BioRad gel dryer. Dried gels were placed directly against Cronex x-ray film (Dupont) for autoradiography.

XI. Nucleic acid hybridization techniques.

DNA was transferred from gels to nitrocellulose membrane (BioRad Transblot Transfer Membrane) by the method of Southern (88).

Recombinant M13 derivatives were denatured and the DNA was adhered to nitrocellulose membranes in the following manner. Three µl of M13 lysates to be screened were spotted on nitrocellulose filters and allowed to air dry. The filters were then treated with each of the following solutions for 5 minutes: i) 0.5 M NaOH, ii) 0.5 M NaOH, 1.5 M

NaCl, iii) twice with 1 M Tris·Cl, pH 7.5 containing 1.5 M NaCl, and iv) 2X SSC. The filters were then air dried followed by baking in vacuo for 2 hours at 80°C.

Radioactive probes were prepared by nick translation according to Rigby et al. (81). α-^{32}P-Deoxyadenosine-5'-triphosphate was purchased from New England Nuclear.

DNA-DNA hybridizations were carried out as follows. Nitrocellulose filters to which DNA had been adhered were first wetted in 6X SSC. Prehybridization was performed by placing the filters in heat-sealable bags containing about 50 ml of hybridization buffer (50% formamide, 4X SSC, and 1X Denhardt's solution (26). The bags were incubated at 42°C with gentle shaking (75-100 rpm) for 1 hour. The prehybridization buffer was poured out through a cut corner of the bags and fresh hybridization buffer containing 25 µg/ml sonicated, denatured calf thymus carrier DNA was added (50 µl/cm^2). Radioactive probes were denatured by heating in boiling water bath for 10 minutes and then placing immediately on ice for 5 minutes before addition to the bags. Hybridization was carried out at 42°C overnight with gentle shaking. Filters were then washed with gentle rocking at room temperature 3 times for 20 minutes with 2X SSC containing 0.1% SDS and 2 times for 30 minutes with 1X SSC containing 0.1% SDS. The filters were air dried, mounted on 3 mm paper, and covered with Saran wrap. Autoradiography was carried out by exposing x-ray film using an intensifying screen at -70°C.

XII. DNA sequencing methodology.

Fragments of the aryletherase gene generated by restriction digestion or sonication (25) were cloned into RF DNA of the M13 vectors mp18 or mp19. Transformation of JM103 was as described (47). Recombinant phage were screened by nucleic acid hybridization with nick translated pNC1. Templates were prepared from positive clones as described eariler. DNA sequencing was then carried out by the methods of Sanger (83) using ddNTPs (P.L. Biochemicals), dNTPs (Boehringer Mannheim), Klenow (BRL), α-^{35}S-dATP (New England Nuclear) and universal pentadecamer M13 primer (New England Biolabs). Sequencing reactions were analyzed on polyacrylamide gels as described earlier.

Random DNA sequences generated by sonication were ordered using the computer program of Staden (89). Promoter searches were carried out using software of Mulligan et al. (72). Restriction site, amino acid sequence, and hydropathy analyses of DNA sequence data were performed using computer programs designed by Douglas Nichol for use with an Apple IIe computer.

RESULTS

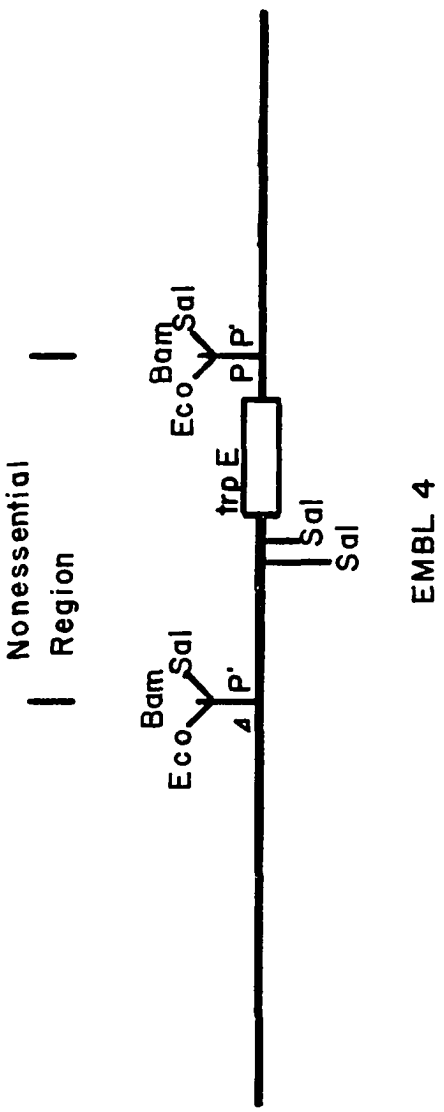
I. Construction of an Erwinia sp. gene library in λ EMBL4.

The lambda replacement vector EMBL4 carries symmetrical polylinkers on each side of the stuffer fragment as shown in Figure 6. Therefore, Sau 3A partial digestion of Erwinia sp genomic DNA was used to create nearly random fragmentation of DNA with cohesive ends compatible for ligation into the Bam HI site of the vector. Selection of recombinant phages is possible by two different mechanisms. First, removal of the red and gam genes on the middle fragment renders recombinant phages Spi$^-$ and allows for direct selection on a P2 lysogen (NM539). Second, simultaneous cleavage of vector DNA with Bam HI and Sal I inactivates the middle fragment and isopropanol precipitation leaves the short cohesive fragments in the supernatant, thereby eliminating religation of the middle fragment to the arms.

In constructing a gene library of Erwinia sp. genomic DNA was necessary to establish the conditions for partial digestion of the DNA. Digestion of 1 μg of DNA with 0.25 U of Sau 3A for one hour yielded the most DNA fragments in the 10-20 kb size range.

Bam HI - Sal I digested EMBL4 and partially digested Erwinia sp. DNA were tested-ligated in molar ratios (arms:inserts) of 0.5:1, 1:1, and 4:1. Molar ratios of 1:1 or 2:1 yielded the highest titers of recombinant phage, typically 1-2 x 10^6 pfu/μg of Erwinia sp. DNA when plated on NM538. Plating on the P2 lysogen (NM539) resulted in pinpoint plaques that were difficult to count and manipulate. When packaging extracts alone were plated on NM538 small, background plaques of about 10^4 pfu/ml were observed.

Figure 6. Schematic structure of the lambda replacement vector EMBL4.



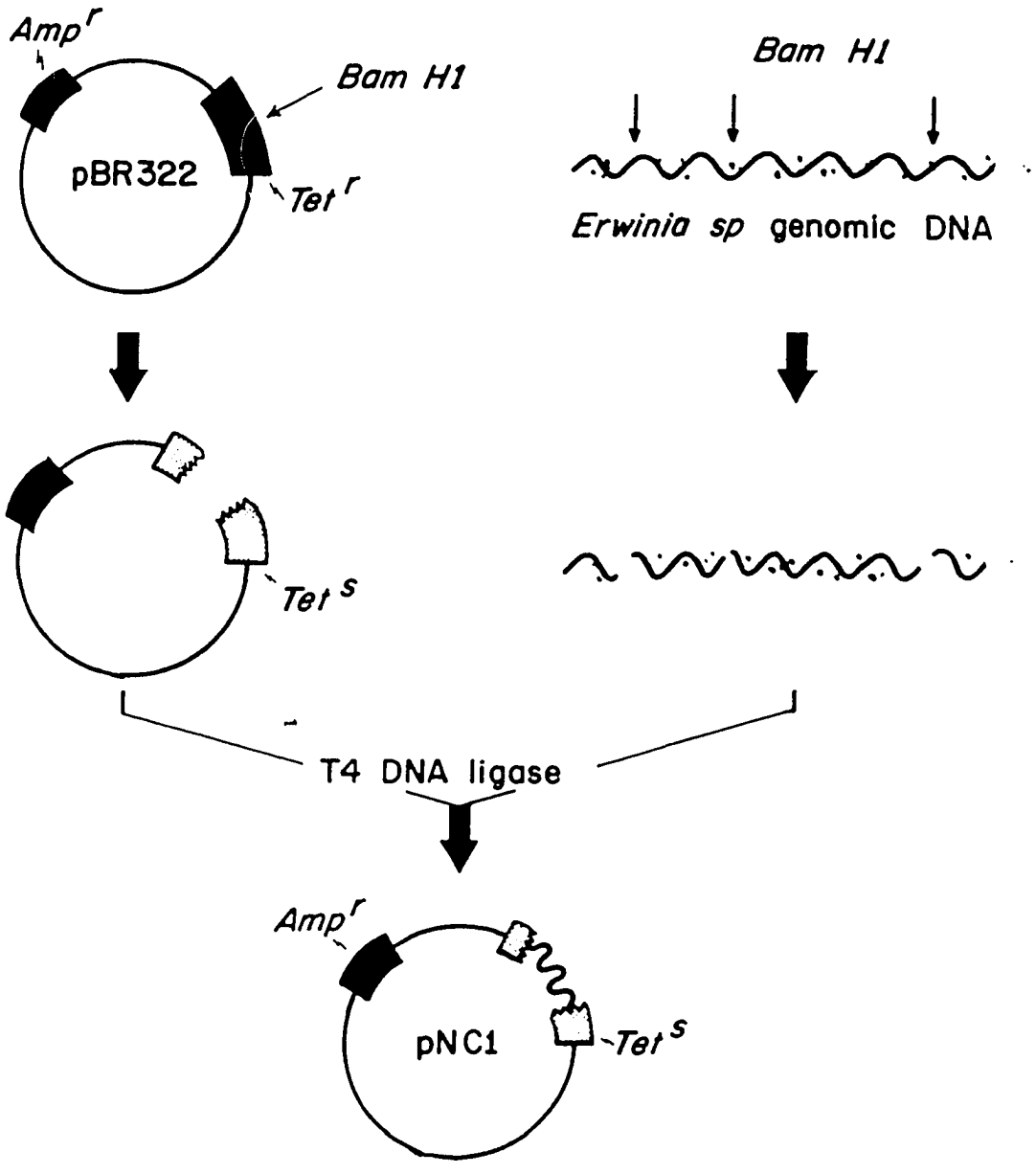
Although the Erwinia sp. is unable to grow on cellobiose as a carbon source, Y. Chon (8) has shown the partially purified aryletherase to be active against chromogenic dyes containing an aromatic ring linked to glucose through a hemiacetal bond, which bears similarity to an ether bond. Such bonds can be thought of as pseudo ether bonds. Thus, aromatic glucosides such as p-nitrophenyl-β-D-glucopyranoside (PNPG) or 5-bromo-4-chloro-3-indoyl-β-D-glucopyranoside (x-glu) serve as substrates for the enzyme, possibly with the aromatic nucleus interacting with the enzyme recognition site. However, repeated attempts to identify the gene encoding the aryletherase by plating recombinant EMBL4 phages on NM538 in the presence of x-glu were unsuccessful.

II. Cloning of the aryletherase gene into E. coli plasmid pBR322.

The general stratagem used to clone the aryletherase gene into pBR322 is depicted in Figure 7. Erwinia sp. DNA was partially digested with 0.5 U Bam HI per μg for one hour to give fragments in the 5-10 kb size range. These fragments were ligated into Bam HI digested pBR322 and then transformed into E. coli Cs412. The transformed cells were plated on selective M9 minimal medium containing ampicillin and 0.5% salicin as the sole carbon source. The structure of salicin is shown in Figure 8. The compound is composed of a salicyl alcohol moiety linked by a hemiacetal bond to glucose. Since Cs412 is unable to grow on salicin, transformants arising on the selective medium must break the hemiacetal (pseudoether) bond to release the glucose moiety for use as a carbon source by the E. coli host. Several hundred colonies appeared after 72 hours incubation at 37°C. Replica plating of 30 on LB medium

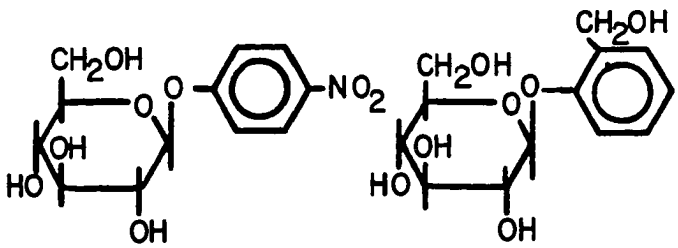
Figure 7. Shotgun cloning of the aryletherase gene from a ligninolytic *Erwinia* sp. into pBR322.

Cloning of the *arylsetherase* gene

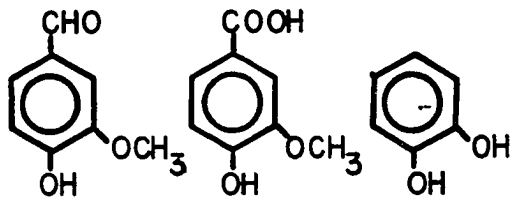


Selection of *Amp r, Tet s* transformants of *E. coli* Cs 412 on salicin minimal medium

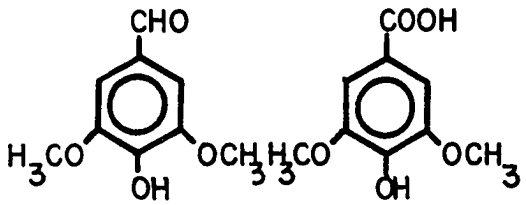
Figure 8. Compounds used in studies of the cloned aryletherase. p-Nitrophenyl-β-D-glucopyranoside (PNPG) (A), salicin (B), vanillin (C), vanillic acid (D), catechol (E), syringaldehyde (F), syringic acid (G), and p-phenoxyphenol (H).



A B



C D E



F G

H

containing ampicillin and tetracycline demonstrated the tetracycline sensitivity of the transformants. Of the 30 isolates, three had the ability to release p-nitrophenol from PNPG after several hours incubation at 37°C. These clones were maintained on M9 minimal medium containing 0.5% salicin and ampicillin for further analysis.

III. Functional analysis of putative clones carrying the aryletherase gene.

Thin layer chromatography (TLC) was used to investigate the ability of the putative clones to degrade kraft lignin and various lignin model compounds shown in Figure 8. The aryletherase was expressed in E. coli Cs412 and demonstrated the following activities. All three transformants oxidized vanillin to vanillic acid. Vanillic acid was then apparently decarboxylated and demethoxylated to yield catechol (Figure 9). Further TLC analysis of one transformant which harbors plasmid pNC1, showed that the cloned aryletherase was able to degrade p-phenoxyphenol, syringaldehyde, and syringic acid. Products of model compound transformations were identified by comparison with standards cochromatographed with the reaction supernatants (Figure 10). The product of p-phenoxyphenol degradation was identified as hydroquionone by TLC. NMR analysis of the p-phenoxyphenol degradation product showed it to be p-benzoquinone, which probably results from the oxidation of hydroquinone. With kraft lignin as a substrate, pNC1 transformants produced at least one unidentified aromatic compound in 2 hours incubation time as shown in Figure 11.

Figure 9. Separation of vanillin and vanillic acid after 4 hours of incubation with the putative clones, 4, 5, and 9 by thin layer chromatography. Control (C), E. coli Cs412 incubated with the appropriate model compound. Abbreviations: standards (st), vanillic acid (VA), vanillin (V), catechol (CA), protocatechualdehyde (PCHO), and protocatechuic acid (PCOOH). Solvent system: Benzene, propionic acid, water (2:1:1). Products and standards were detected by illumination with ultraviolet light.

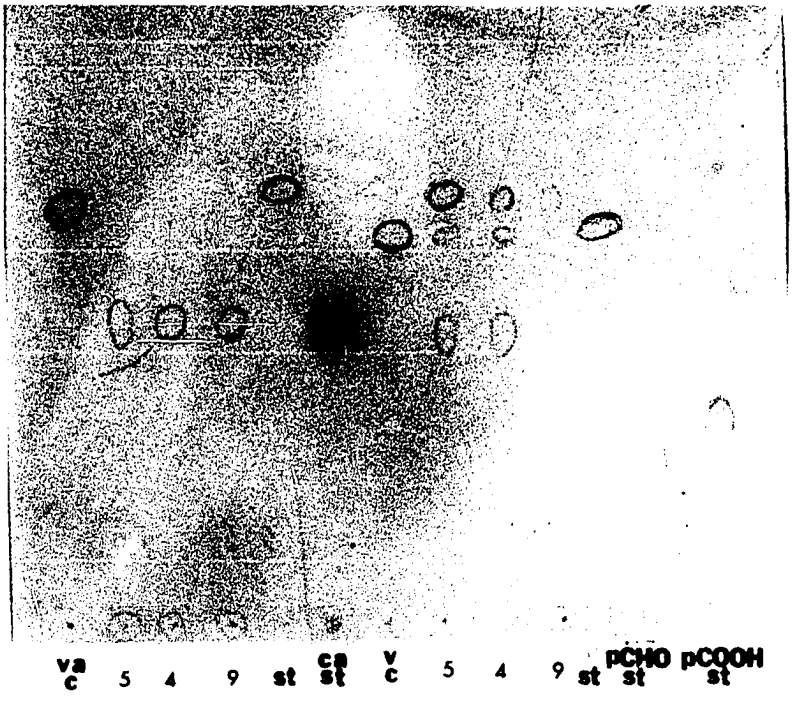


Figure 10. Separation of metabolites of model compounds after 4 hours incubation with Cs412 transformed with pNCl (A) or Cs412 as a control (B). Abbreviations: p-phenoxyphenol (ppp), catechol (ca), hydroquinone (hq), resorcinol (r), syringaldehyde (sCHO), and syringic acid (sCOOH). Solvent system: Benzene, 2-propanol, NH_4OH (4:1:1). Detection solution: diazotized sulfanilic acid.

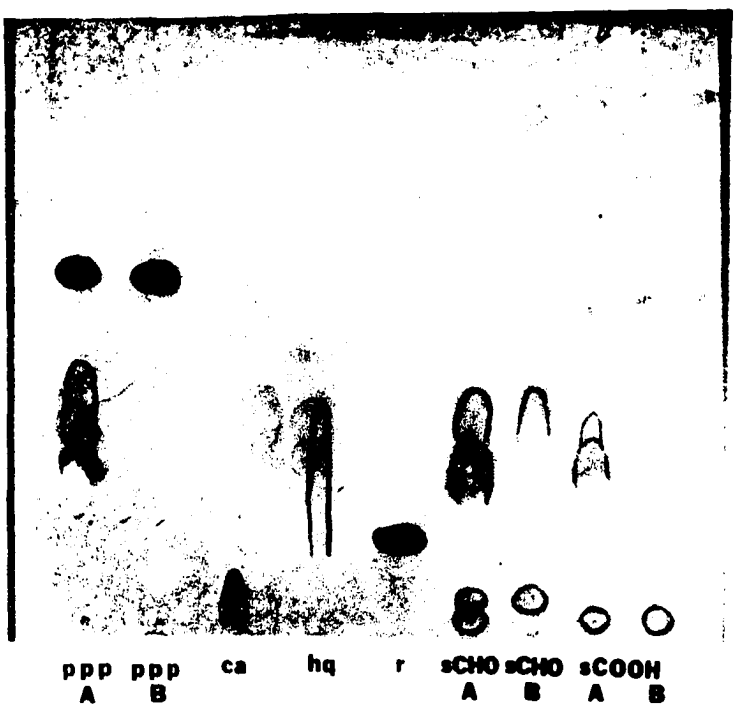
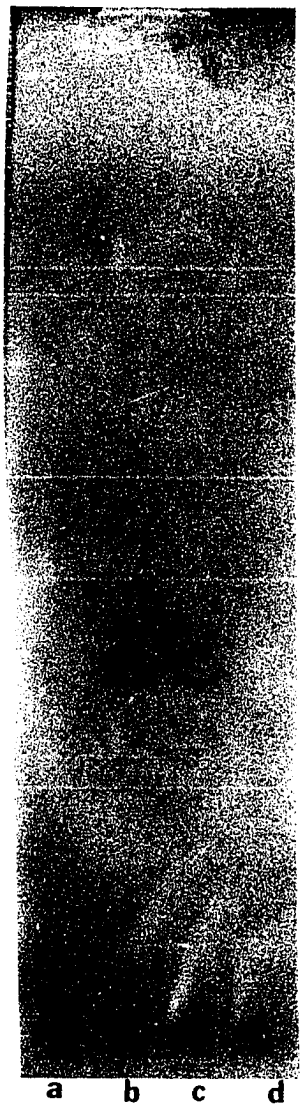


Figure 11. Separation of an aromatic lignin degradation product after 2 hours of incubation with Cs412 as a control (a), Cs412/pNC2 (b), and Cs412/pNC1 (c), and lignin standard (d). Solvent system: Benzene, 2-propanol, NH_4OH (4:1:1). Compounds were detected by illumination with ultraviolet light.



IV. Plasmid analyses.

a. Rapid plasmid screening.

Two of the three putative clones were shown to harbor derivatives of pBR322 using the rapid plasmid preparation technique of Birnboim and Doly (4). These plasmids were designated pNC1 and pNC2. Large quantities were prepared on CsCl gradients for further analysis.

b. Retransformation.

Transformation of E. coli Cs412 with either pNC1 or pNC2 conferred the ability to grow on M9 medium containing salicin as the carbon source and ampicillin as the other selective agent. Colonies appeared after 48 hours at 37°C. Efficiency of transformation was about 1×10^4 cfu per μg of plasmid DNA. Controls using pBR322 did not grow on the selective medium.

c. Dot blot hybridization.

Proof that pNC1 and pNC2 are derivatives of pBR322 which contain inserts of Erwinia sp. DNA was obtained in dot blot hybridization experiments. Both recombinant plasmids hybridized to varying amounts of Erwinia sp. genomic DNA. Nonspecific hybridization to calf thymus DNA was not observed. The results for pNC1 are presented in Figure 12.

d. Restriction enzyme mapping.

Restriction enzyme mapping of pNC1 was performed using a variety of endonucleases which recognize unique hexanucleotide sequences and have a single site in pBR322. Restriction digests were analyzed on horizontal agarose gels (Figure 13). Drigest molecular weight markers were used to construct a standard curve of mobility vs. $\log_{10}$ nulceotide base pairs as shown in Figure 14. In this manner the data presented in Table 1 was

Figure 12. Dot blot hybridization of ^{32}P-labeled pNC1 with Erwinia sp. genomic DNA.

Erwinia sp. DNA

calf thymus DNA

10 5 2.5 1

µg DNA / spot

Figure 13. Restriction enzyme digests used to map pNC1. Data is presented in Table 1. pNC1 digested with: Bam HI (a), EcoRI (b), Hind III (c), Sal I (d), Pst I (e), Pvu II (f), Ava I (g), and Cla I (h). Drigest molecular size markers are included as standards (sizes are kbp).

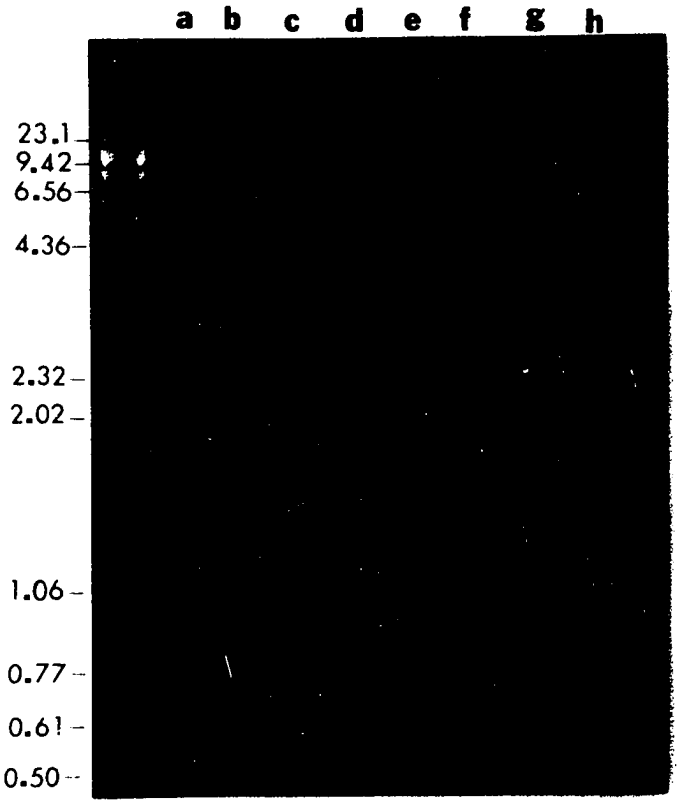


Figure 14. Standard curve ($\log_{10}$ kbp vs. migration in a 0.7% agarose gel) of data presented in Table 1 used to determine the size of restriction fragments of pNCl.

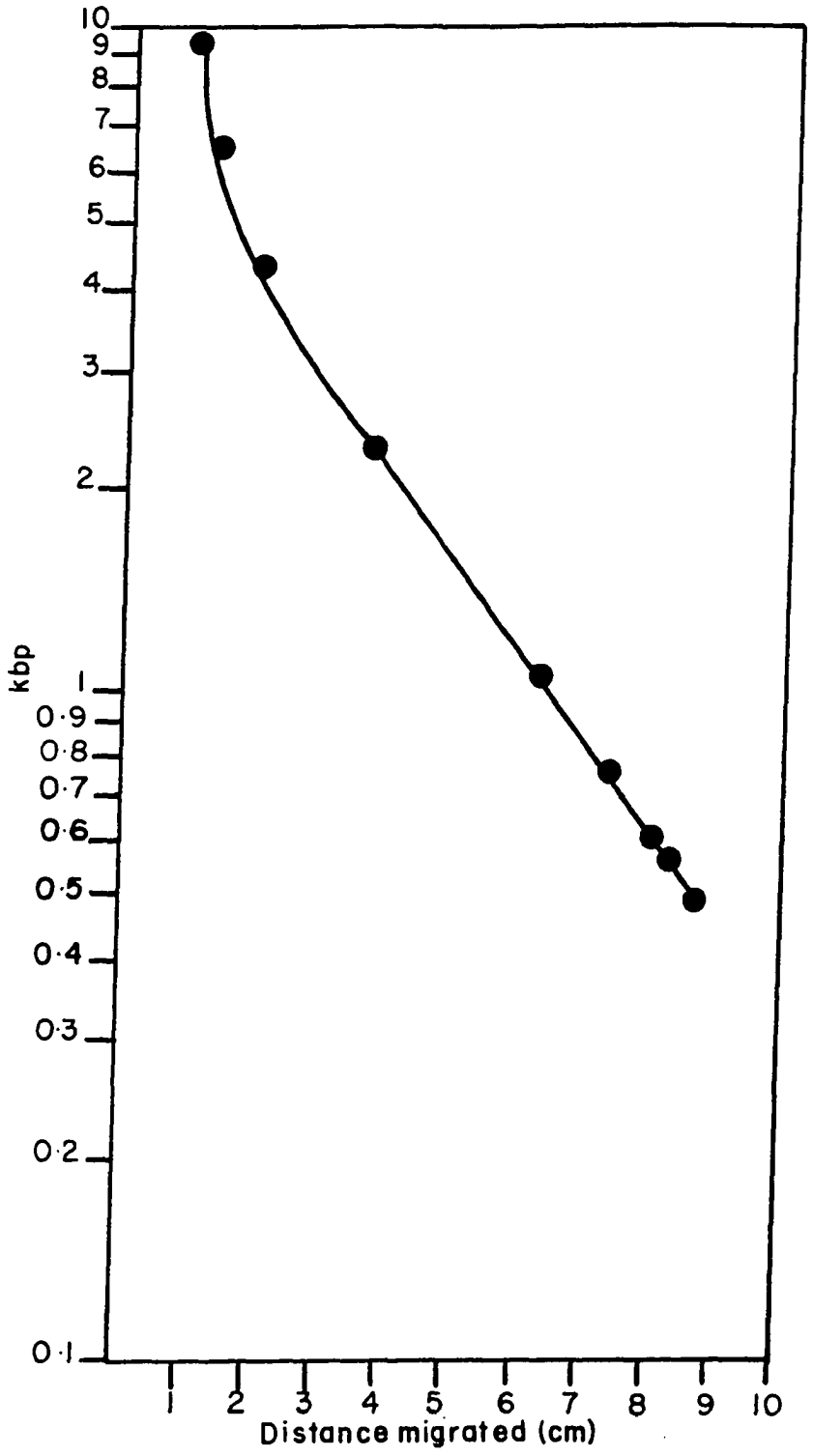


Table 1. Data for determination of the molecular size and restriction map of pNCl.

	Distance migrated (cm)	bp
Drigest molecular size markers	1.00	9416
	1.35	6557
	2.00	4361
	3.75	2322
	4.15	2027
	6.35	1057
	7.40	770
	8.05	609
	8.30	564
	8.70	495
pNCl (Eco RI)	1.70	5110*
pNCl (Bam HI)	2.00	4361*
	7.10	810*
pNCl (Sal I)	1.95	4520*
	8.10	590*
pNCl (Pst I)	2.75	3200*
	4.35	1910*
pNCl (Pvu II)	2.95	3000*
	4.05	2100*

* calculated from standard curve.

used to construct the restriction map depicted in Figure 15. No internal restriction sites were observed for the enzymes EcoRI, Hind III, Ava I, or Cla I. The results of the restriction enzyme mapping were confirmed by nucleotide sequence data (see below).

The size of the Bam HI generated insert containing the aryletherase gene was estimated to be 810 bp based upon its mobility in agarose gels.

Interestingly, a restriction map could not be deduced for pNC2. pNC2 is an approximately 9.4 kb plasmid. The patterns of restriction were similar to those obtained for pNC1, i.e., pNC2 contained restriction sites for the same enzymes that cut pNC1. However, the 810 bp Bam HI fragment could not be excised from pNC2. Hybridization of Southern blots of restriction digests of pNC2 with either nick-translated pBR322 (Figure 16) or the 810 bp DNA fragment from pNC1 (Figure 17) also produced anomolous results. All restriction fragments hybridized with both probes. However, the hybridization was not nonspecific since the λ Hind III molecular weight markers did not hybridize with either probe. No explanation of these results can be based on the existing data.

V. Southern transfer analysis of Erwinia sp. genomic DNA.

^{32}P-nick translated pNC1 was used as a probe to demonstrate sequence homology between the cloned gene and Erwinia sp. genomic DNA digested with several restriction endonucleases (Figure 18). Since it was known that the gene does not contain any internal Hind III sites, Erwinia sp. genomic DNA was digested with Hind III and Bam HI to enhance the signal of the 810 bp DNA fragment which contains the gene. The other enzymes were used in single digestions. The radiolabeled probe

Figure 15. Partial restriction enzyme map of pNCl deduced from the data in Table 1.

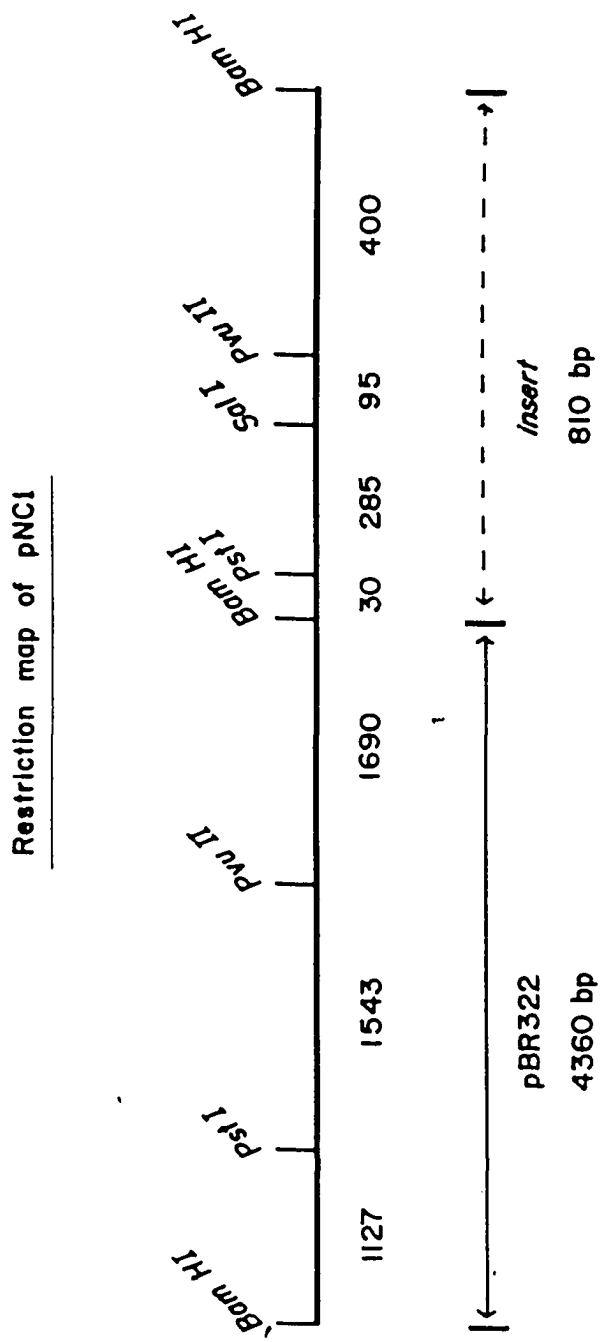


Figure 16. A. Restriction enyzme digests of pNC2 run on a 0.7% agarose gel: Bam HI + Eco RI (a), Bam HI (b), Bam HI + Sal I (c), Bam HI + Hind III (d), and Bam HI + Pst I (e). Hind III digested λ DNA is included as molecular size markers.

B. Southern transfer of DNA from gel in 16A hybridized with ^{32}P-labeled pBR322.

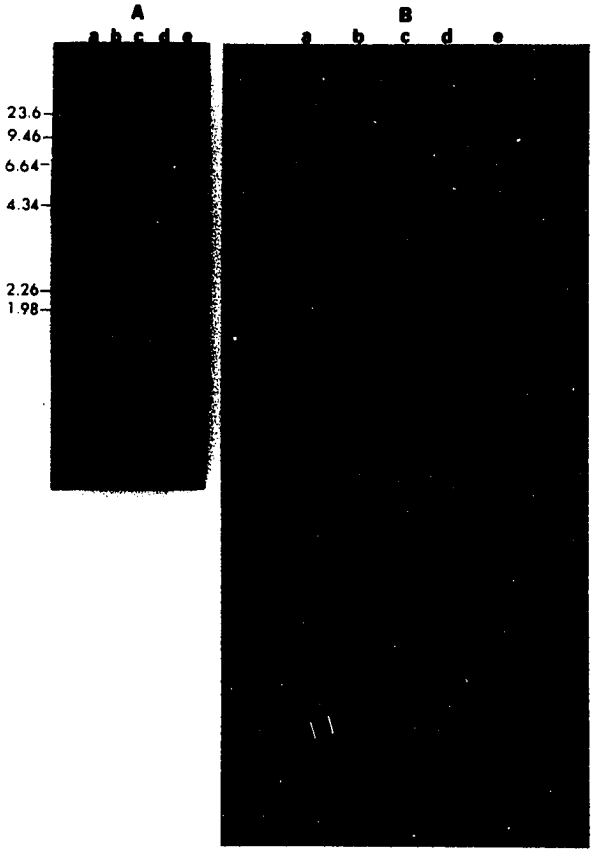


Figure 17. A. Restriction enzyme digests of pNC2 run on a 0.7% agarose gel: Bam HI (a), Bam HI + Eco RI (b), Bam HI + Sal I (c), Bam HI + Hind III (d), and Bam HI^{+} Pst I (e). Hind III digested λ DNA is included as molecular size markers.

B. Southern transfer of DNA from gel in 17A hybridized with ^{32}P-labeled 810 bp insert from pNC1.

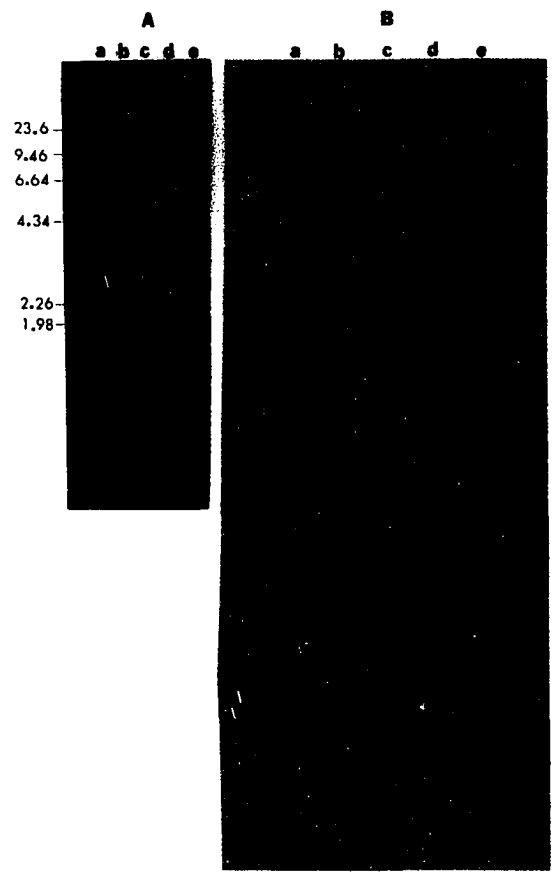
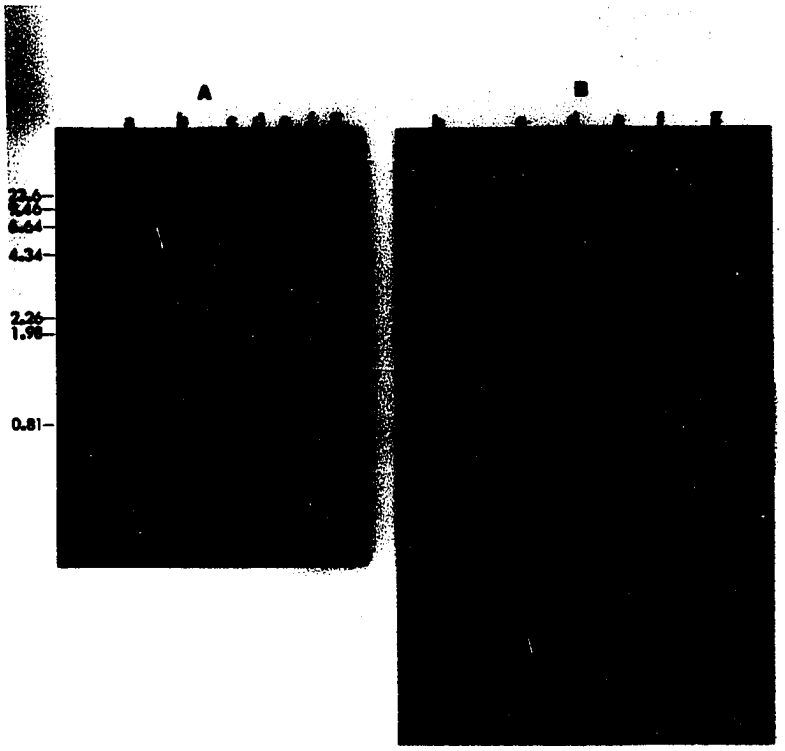


Figure 18. A. Agarose (0.7%) gel electrophoresis of the 810 bp Erwinia sp. insert from pNC1 (a, b) and Erwinia sp. genomic DNA digested with Bam HI + Hind III (c), Eco RI (d), Sal I (e), Hind III (f), and Pst I (g). Hind III digested DNA is included as molecular size markers.

B. Southern transfer of DNA from gel in 18A hybridized with ^{32}P-labeled pNC1. The 810 bp insert from pNC1 is included in "b" as a molecular size marker.



showed hybridization to a single Bam HI fragment which migrated with the same mobility as the 810 bp Bam HI fragment encoding the aryletherase gene. The probe also hybridized with single Hind III and Pst I fragments, as well as with 2 Sal I fragments. On examination of the restriction map of the aryletherase gene these results suggest that the gene is present as a single copy on the Erwinia sp. chromosome.

VI. Cloning the the E. coli lac Z gene into pNC1.

The E. coli lac Z gene encoding β-galactosidase was excised from λgt4lac5 DNA on a 6.6 kb EcoRI fragment. This fragment was subcloned into the EcoRI site of pNC1 to yield pNC3 as shown in Figure 19. pNC3 was used to transform E. coli strain Rc30 (lac^-). Lac^+ transformants were selected on MacConkey medium containing ampicillin as red colonies after 48 hours. Transformants containing pNC3 may be useful in developing a bioconversion process for lignin (see Discussion).

VII. Cloning of the aryletherase gene into the expression vector pCQV2.

The expression vector pCQV2 (Figure 20) was used in an attempt to increase cellular levels of the aryletherase gene product in E. coli Cs412. This expression vector contains a region of DNA represented by lambda early promoter P_R-ribosome binding site-ATG-GATCC (78). P_R is under control of the temperature-sensitive CI857 deriviative of the CI repressor. Therefore, any gene on a Bam HI fragment can be inserted just downstream from the ATG start codon and induced for expression at 42°C. The Bam HI fragment containing the aryletherase gene was ligated into the Bam HI site of phosphatase treated pCQV2. However, induction of transformants at 42°C failed to produce PNPG activity. One subclone was digested with Bam HI and randomly religated in hope of obtaining the

Figure 19. Schematic representation of pNC3 indicating the insertion of the 6.6 kbp lac5 Eco RI fragment into pNC1.

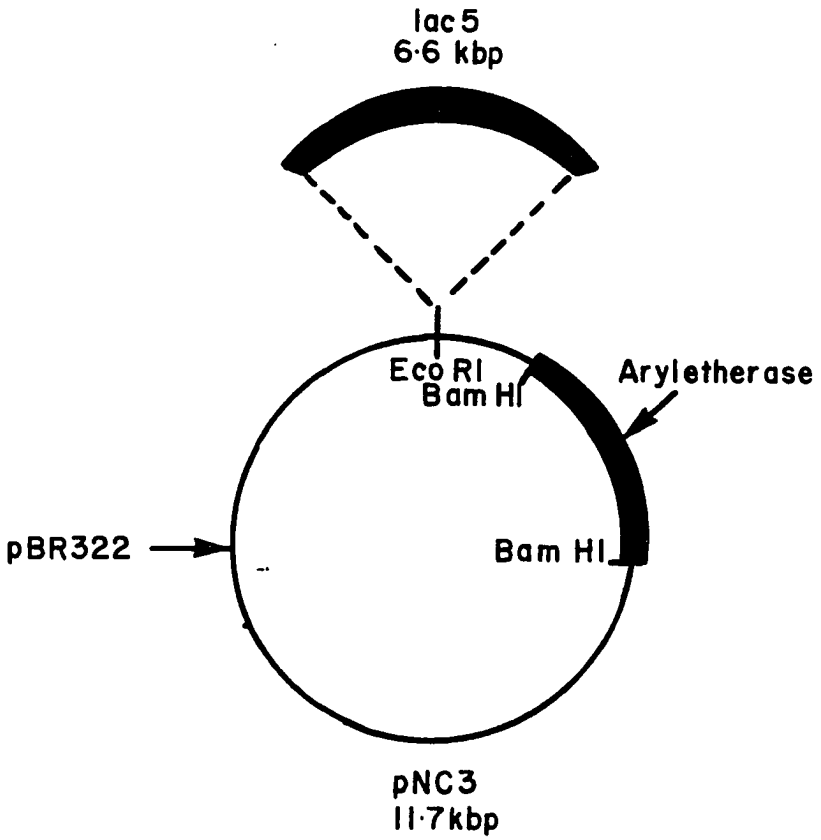
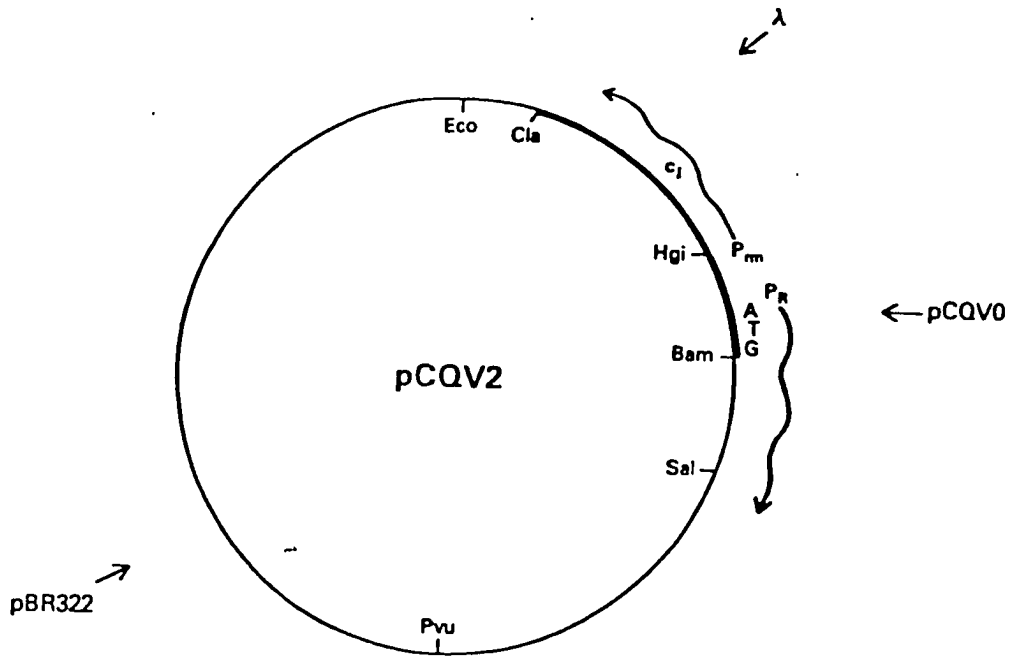


Figure 20. Schematic representation of the expression vector pCQV2 (78).



arylether ase gene in both orientations. Induction of 20 transformants containing the 810 bp fragment still failed to yield any clones with PNPG activity. DNA sequence data obtained later showed that the gene was inserted into pCQV2 out of translational reading frame.

VIII. Dideoxyribonucleotide sequencing of the arylether ase gene.

The nucleotide sequence of the Bam HI insert from pNC1 carrying the arylether ase gene was determined by the dideoxyribonucleotide chain termination method of Sanger et al. (83). The strategy used to sequence the arylether ase gene is as follows. DNA fragments 200-400 bp in length generated by sonication of the gene in M13 mp18 replicative form DNA were subcloned back into M13 mp18. Phage which formed white plaques on JM103 on M9 minimal medium in the presence of x-gal contained DNA inserted into the region of the vector encoding the β-galactosidase α-complementing peptide. These recombinant phage, which typically represented about 10% of the total phage population, were screened by dot blot hybridization to ^{32}P-nick translated pNC1 as shown, for example, in Figure 21. Positive clones were used in sequencing reactions. Also, restriction fragments generated by double digestion with Bam HI and either Sal I or Bgl II were cloned into both M13 mp18 and M13 mp19 to obtain sequence data on both DNA strands from these sites. As a result, an array of overlapping DNA sequence data was generated and ordered by computer analysis (89) as shown in Figure 22. On the whole, 792 base pairs were sequenced on both strands of the DNA. The sequence of the entire Bam HI insert from pNC1 carrying the arylether ase gene is shown in Figure 23.

Figure 21. Identification of recombinant M13mp18 phage spotted on nitrocellulose filters which contain aryletherase sequences by hybridization with ^{32}P-labeled pNC1. In this example, 9 of 75 recombinants screened initially for inactivation of β-galactosidase exhibited homology with the probe. M13mp18 was spotted as a negative hybridization control beneath the arrow.

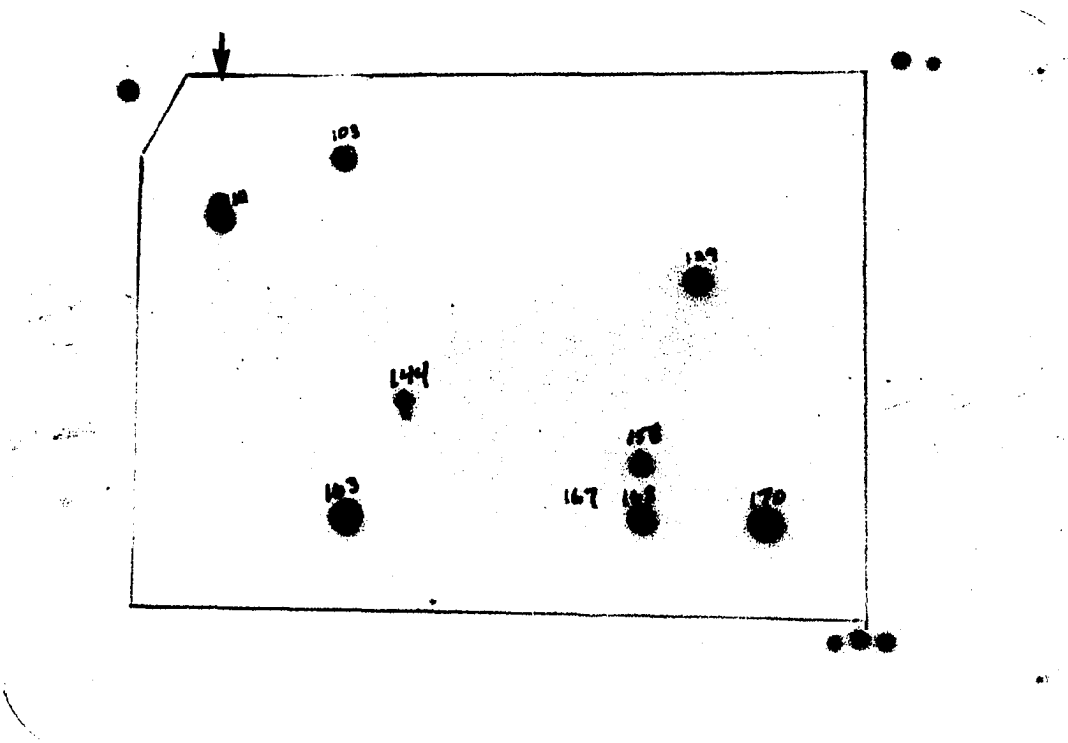


Figure 22. Strategy used to sequence the aryletherase gene.

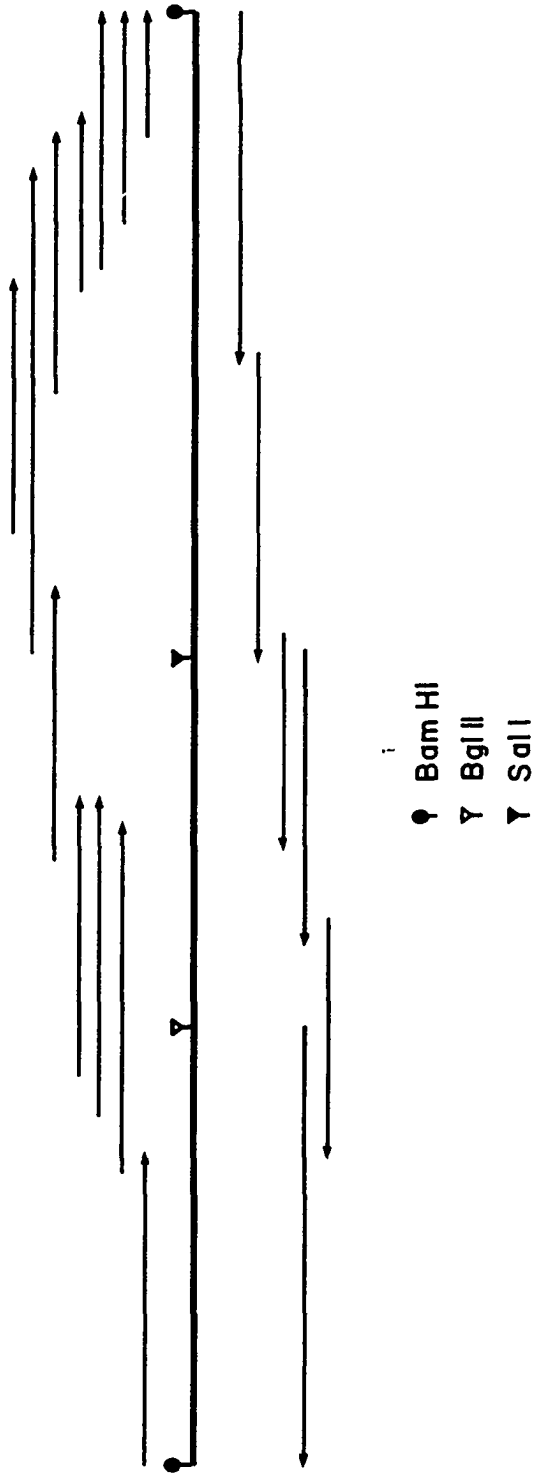


Figure 23. Nucleotide sequence of the aryletherase gene beginning with the 5' end proximal to the tet^r gene promoter.

TOTAL NUCLEOTIDES = 792

```
        10         20         30         40         50         60         70
.........! .........! .........! .........! .........! .........! .........!
GGATCCCAGC TAGSACAGTC ATGGAGCGCC GTAGGCATCA TACGTACGGT AGAGTCGAGC GAGCATGCTG

        80         90        100        110        120        130        140
.........! .........! .........! .........! .........! .........! .........!
CCACCCTAGC GTAGGCATTG TCAGCGCGCT TCTTCGTCCG GCAGCTAGGC GGCCAACATC TAATATCCGT

       150        160        170        180        190        200        210
.........! .........! .........! .........! .........! .........! .........!
CCGGCATCAG CCCAGGGCTG CTTACCGAGC CTGTTCAACA TCCGATCAGG TGATCGAAGT GTTCGTCATC

       220        230        240        250        260        270        280
.........! .........! .........! .........! .........! .........! .........!
GGCGTCGGCG GCGTCGGCGG GGCGCTGATC GAGCAGATCT ACCGCCAGCA GCCGTGGCTG AAGCAAAAAC

       290        300        310        320        330        340        350
.........! .........! .........! .........! .........! .........! .........!
ATATCGACCT GCGGGTGCTG CGGCATCGCC AACTGCGCGT CGATGCTCGA CAGTGCAGGC ATCGCGCTGG

       360        370        380        390        400        410        420
.........! .........! .........! .........! .........! .........! .........!
CAGCTGCGAC GAGCTGGCCG GCGCGCAGGA GCCGTTCAAT CTCGGCCGCC TGATCCGGCT GGTGAAGGAG

       430        440        450        460        470        480        490
.........! .........! .........! .........! .........! .........! .........!
TATCACCTGC TGAACCCGGT GATCGTCGAC TGTACCTCCA GCCAGGCGTG GCCGATCAGT ATGTGGACTT

       500        510        520        530        540        550        560
.........! .........! .........! .........! .........! .........! .........!
CCTGGCGGAC GGCTTCCATG TGGTAACGCC GCAACAAAAA GGCCAACTAC CTCGTCGATG AACTATTATC

       570        580        590        600        610        620        630
.........! .........! .........! .........! .........! .........! .........!
AGCAACTGCG CGCCGCCGCC GCCGGTTCGC ACGCAAGTTC CTCTAACGAC ACAACGTCGG CGCGGTCTGC

       640        650        660        670        680        690        700
.........! .........! .........! .........! .........! .........! .........!
CGTGATCGAG ACCTGCAAAA CTGCTGACGC GGCGATGACG TGGTGCGTTT CTCCGGCATC CTGTCCGGTT

       710        720        730        740        750        760        770
.........! .........! .........! .........! .........! .........! .........!
CGCTGTCCTT TATTTTTGGC AAGCTGGATG AAGGGCTGTC GTGTCGGCGG CGACTCTGCA GGCCAGAGCA

       780        790        800        810        820        830        840
.........! .........! .........! .........! .........! .........! .........!
ATGGCTACAC CGAGCCGGAT CC
```

IX. Analyses of the DNA sequence of the aryletherase gene.

Restriction enzyme site analysis of the DNA sequence of the Bam HI fragment carrying the aryletherase gene was performed using the computer program of Douglas Nichol. The results of this computer search are shown in Figure 24. The restriction site identified enzymatically (Bgl II, Pvu II, Pst I, and Sal I) were confirmed by the DNA sequence data. Also, enzymes failing to cut the aryletherase gene (Ava I, Cla I, Eco RI, and Hind III) were predicted not to have recognition sites by the DNA sequence data.

Translational analysis of the DNA sequence identified an open reading frame beginning with an ATG start codon at position 21. The open reading frame has the capacity to encode 175 amino acids before a putative stop codon is encountered, as shown in Figure 25. This corresponds to a polypeptide of approximately 21,000 daltons.

Furthermore, the DNA sequence at -10 from the ATG start codon is UAGGA. This sequence is found in most Shine-Delgarno ribosome binding sites examined to date (36).

A computer search for promoters with homology to the E. coli concensus sequence failed to identify any putative promoters. Indeed, from the DNA sequence data it is apparent that the Bam HI DNA fragment does not possess the capacity to encode up-stream regulatory regions of the gene.

Computer evaluation of the hydrophilicity and hydrophobicity of the predicted amino acid sequence is shown in Figure 26.

Figure 24. Restriction enzyme recognition site analysis of the aryletherase gene nucleotide sequence.

AATI GACGTG

667

ACCI GTVSAC

445

AFLII CTTAAG

*** NO MATCH ***

AFLIII ACUYGT

*** NO MATCH ***

AHAII GUCGYC

211

220

AHAIII TTTAAA

*** NO MATCH ***

ALUI AGCT

8

113

352

362

722

API GGGCCC

*** NO MATCH ***

ASUII TTCGAA

*** NO MATCH ***

AVAI CYCGUG

*** NO MATCH ***

AVAII GGWCC

*** NO MATCH ***

AVAIII ATGCCAT

*** NO MATCH ***

AVRII CCTAGG

*** NO MATCH ***

BALI TGGCCA

*** NO MATCH ***

BAMHI GGATCC

1

BANI GGYUCC

*** NO MATCH ***

BBVI GCAGC

111

258

350

BCLI TGATCA

*** NO MATCH ***

BGLI GCCNNNNNGGC

*** NO MATCH ***

BGLII AGATCT

245

BSP1386 GNGCNC

24

94

230

371

568

BSSHII GCGCGC

94

371

568

BSTEII GGTNACC

*** NO MATCH ***

BSTNI CCWGG

152

462

491

BSTXI CCANNNNNNTGG

763

CLAI ATCGAT

*** NO MATCH ***

DDEI CTNAG

*** NO MATCH ***

ECORI GAATTC

*** NO MATCH ***

ECORV GATATC

*** NO MATCH ***

FNUDII CGCG

95

316

343

372

569

621

658

FNU4H GCNGC

67

111

119

157

218

258

297

300

350

353

395

518

572

575

578

659

747

FOKI GGATG

726

HAEI WGGCCW

530

760

HAEII UGCGCY

25

231

HAEIII GGCC

121

366

394

470

531

761

HGAI GACGC

656

HGIAI GWGCWC

*** NO MATCH ***

HHAI GCGC

26

94

96

232

315

344

371

373

568

570

620

HINCII GTYUAC

445

HINDIII AAGCTT

*** NO MATCH ***

HINFI GANTC

52

752

HPHI GGTGA

189

411

438

HPAI GTTAAC

*** NO MATCH ***

HPAII CCGG

108

141

368

405

436

582

683

695

785

KPNI GGTACC

*** NO MATCH ***

MBOI GATC

2

184 402

192 441

237 474

246 634

MBOII GAAGA

*** NO MATCH ***

MLUI ACGCGT

*** NO MATCH ***

MNLI CCTC

455

540

600

MSTI TGCGCA

*** NO MATCH ***

MSTII CCTNAGG

*** NO MATCH ***

NAEI GCCGGC

367

NARI GGCGCC

*** NO MATCH ***

NCII CCZGG

435

NCOI CCATGG

*** NO MATCH ***

NDEI CATATG

*** NO MATCH ***

NRUI TCGCGA

*** NO MATCH ***

PVUI CGATCG

*** NO MATCH ***

PVUII CAGCTG

351

PSTI CTGCAG

756

RSAI GTAC

44

452

SACI GAGCTC

*** NO MATCH ***

SACII CCGCGG

*** NO MATCH ***

SALI GTCGAC

445

SAU961 AGTACT

*** NO MATCH ***

SCAI AGTACT

*** NO MATCH ***

SCRFI CCNGG

152

435

462

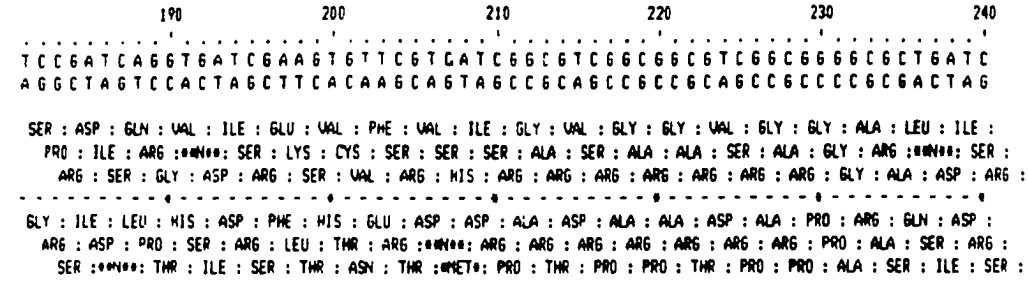
491

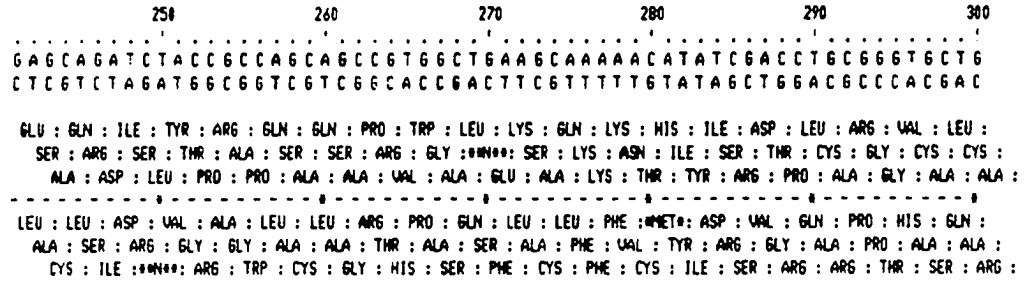
```
SMAI            CCCGGG
  *** NO MATCH ***
SFANI           GATGC
322
SFII            GGCCNNNNNCCGG
  *** NO MATCH ***
SPHI            GCATGC
63
STUI            AGGCCT
  *** NO MATCH ***
TAQI            TCGA
55
194
239
284
320
327
446
545
636
TH111I          GACNNNGTC
  *** NO MATCH ***
XBAI            TCTAGA
  *** NO MATCH ***
XHOI            CTCGAG
  *** NO MATCH ***
XMAI            CCCGGG
  *** NO MATCH ***
XMAIII          CGGCCG
393
SMNI            GAANNNTTC
  *** NO MATCH ***
```

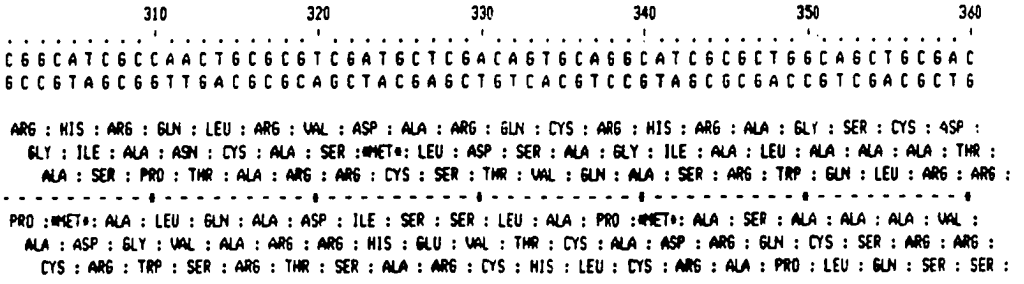
Figure 25. Translational analysis of the aryletherase gene nucleotide sequence.

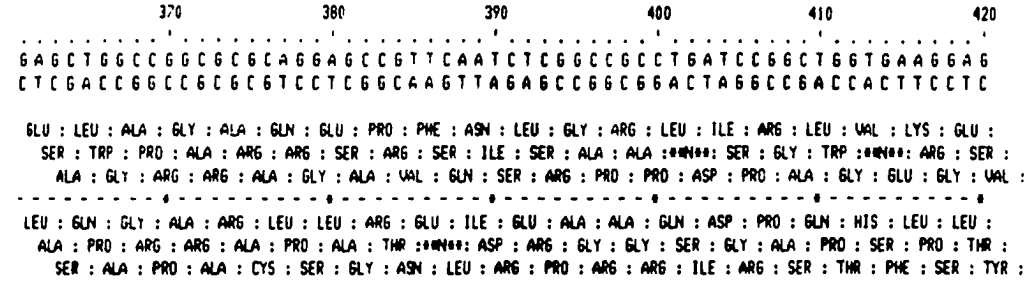
LENGTH =792

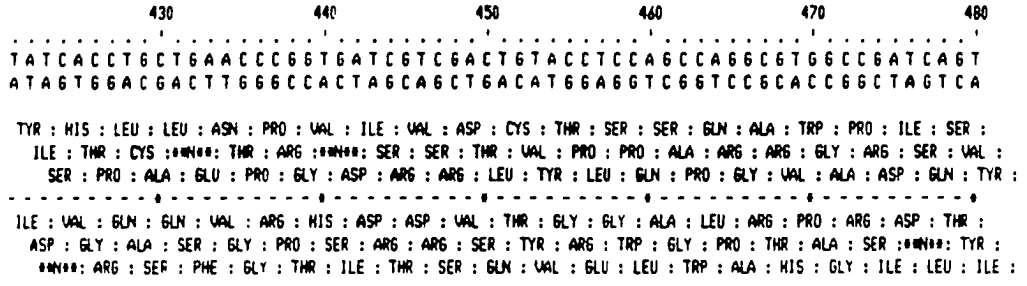
```
                10                  20                  30                  40                  50                  60
. . . . . ' . . . . . ' . . . . . ' . . . . . ' . . . . . ' . . . . . '
G G A T C C C A G C T A G G A C A G T C A T G G A G C S C C G T A G G C A T C A T A C G T A C G G T A G A G T C G A G C
C C T A G G G T C G A T C C T G T C A G T A C C T C G C G G C A T C C G T A G T A T G C A T G C C A T C T C A G C T C G

 GLY : SER : GLN : LEU : GLY : GLN : SER : TRP : SER : ALA : VAL : GLY : ILE : ILE : ARG : THR : VAL : GLU : SER : SER :
   ASP : PRO : SER :*#N**: ASP : SER : HIS : GLY : ALA : PRO :*#N**: ALA : SER : TYR : VAL : ARG :*#N**: SER : ARG : ALA :
     ILE : PRO : ALA : ARG : THR : VAL :#MET*: GLU : ARG : ARG : ARG : HIS : HIS : THR : TYR : GLY : ARG : VAL : GLU : ARG :
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
 SER : GLY : LEU :*#N**: SER : LEU :*#N**: PRO : ALA : GLY : TYR : ALA : ASP : TYR : THR : ARG : TYR : LEU : ARG : ALA :
   ILE : GLY : ALA : LEU : VAL : THR :#MET*: SER : ARG : ARG : LEU : CYS :*#N**: VAL : TYR : PRO : LEU : THR : SER : ARG :
     ASP : TRP : SER : PRO : CYS : ASP : HIS : LEU : ALA : THR : PRO :#MET*:#MET*: ARG : VAL : THR : SER : ASP : LEU : SER :

                70                  80                  90                 100                 110                 120
. . . . . ' . . . . . ' . . . . . ' . . . . . ' . . . . . ' . . . . . '
G A G C A T G C T G C C A C C C T A G C G T A G G C A T T G T C A G C G C G C T T C T T C G T C C G G C A G C T A G G C
C T C G T A C G A C G G T G G G A T C G C A T C C G T A A C A G T C G C G C G A A G A A G C A G G C C G T C G A T C C G

 GLU : HIS : ALA : ALA : THR : LEU : ALA :*#N**: ALA : LEU : SER : ALA : ARG : PHE : PHE : VAL : ARG : GLN : LEU : GLY :
   SER :#MET*: LEU : PRO : PRO :*#N**: ARG : ARG : HIS : CYS : GLN : ARG : ALA : SER : SER : SER : GLY : SER :*#N**: ALA :
     ALA : CYS : CYS : HIS : PRO : SER : VAL : GLY : ILE : VAL : SER : ALA : LEU : LEU : ARG : PRO : ALA : ALA : ARG : ARG :
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
 LEU :#MET*: SER : GLY : GLY :*#N**: ARG : LEU : CYS : GLN :*#N**: ARG : ALA : GLU : GLU : ASP : PRO : LEU :*#N**: ALA :
   ALA : HIS : GLN : TRP : GLY : LEU : THR : PRO :#MET*: THR : LEU : ALA : SER : ARG : ARG : GLY : ALA : ALA : LEU : ARG :
     CYS : ALA : ALA : VAL : ARG : ALA : TYR : ALA : ASN : ASP : ALA : ARG : LYS : LYS : THR : ARG : CYS : SER : PRO : PRO :

                130                 140                 150                 160                 170                 180
. . . . . ' . . . . . ' . . . . . ' . . . . . ' . . . . . ' . . . . . '
G G C C A A C A T C T A A T A T C C G T C C G G C A T C A G C C C A G G G C T G C T T A C C G A G C C T G T T C A A C A
C C G G T T G T A G A T T A T A G G C A G G C C G T A G T C G G G T C C C G A C G A A T G G C T C G G A C A A G T T G T

 GLY : GLN : HIS : LEU : ILE : SER : VAL : ARG : HIS : GLN : PRO : ARG : ALA : ALA : TYR : ARG : ALA : CYS : SER : THR :
   ALA : ASN : ILE :*#N**: TYP : PRO : SER : GLY : ILE : SER : PRO : GLY : LEU : LEU : THR : GLU : PRO : VAL : GLN : HIS :
     PRO : THR : SER : ASN : ILE : ARG : PRO : ALA : SER : ALA : GLN : GLY : CYS : LEU : PRO : SER : LEU : PHE : ASN : ILE :
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
 ALA : LEU :#MET*:*#N**: TYR : GLY : ASP : PRO :#MET*: LEU : GLY : PRO : SER : SER : VAL : SER : GLY : THR :*#N**: CYS :
   GLY : VAL : ASP : LEU : ILE : ARG : GLY : ALA : ASP : ALA : TRP : PRO : GLN : LYS : GLY : LEU : ARG : ASN : LEU :#MET*:
     TRP : CYS : ARG : ILE : ASP : THR : ARG : CYS :*#N**: GLY : LEU : ALA : ALA :*#N**: ARG : ALA : GLN : GLU : VAL : ASP :
```

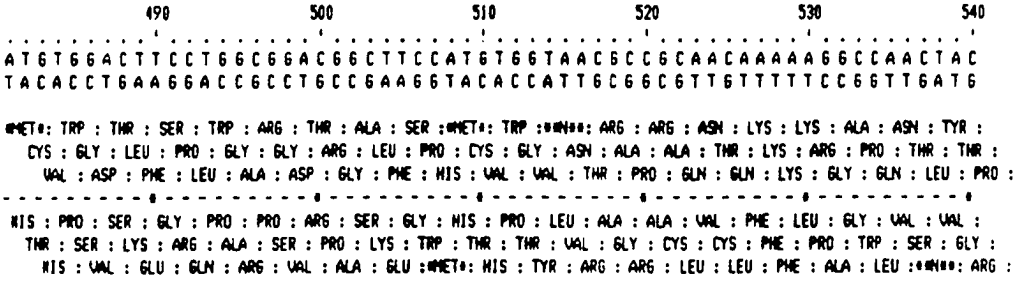


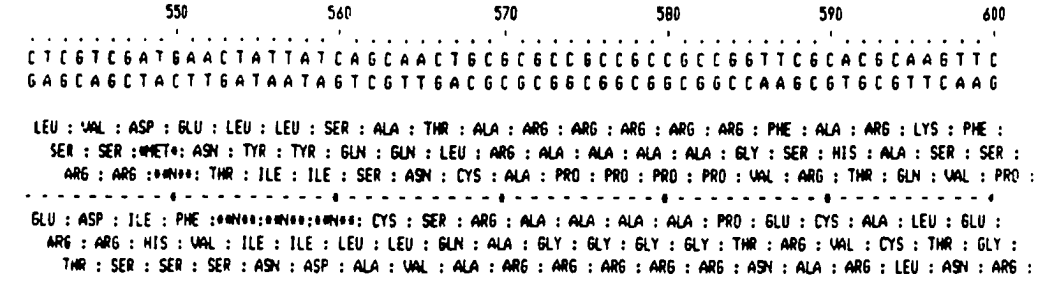


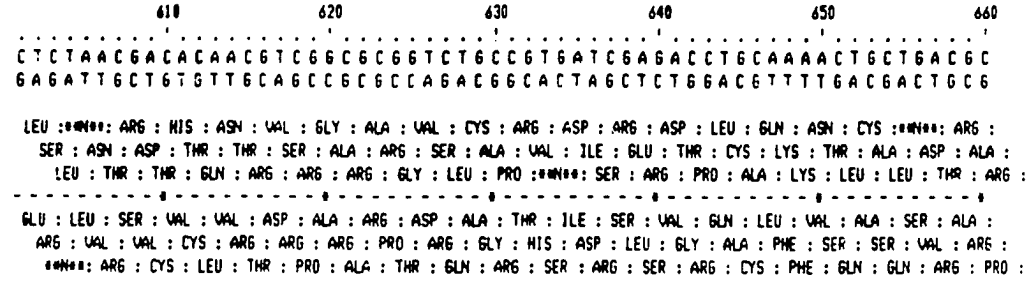


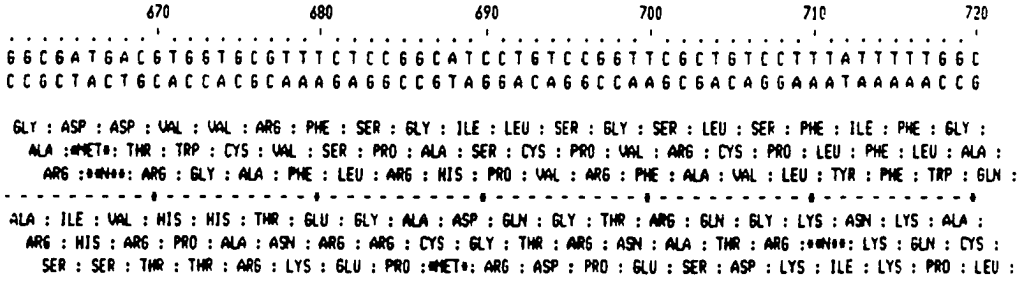












```
            730               740               750               760               770               780
            '                 '                 '                 '                 '                 '
AAGCTGGATGAAGGGCTGTCGTGTCGGCGGCGACTCTGCAGGCCAGAGCAATGGCTACAC
TTCGACCTACTTCCCGACAGCACAGCCGCCGCTGAGACGTCCGGTCTCGTTACCGATGTG

 LYS : LEU : ASP : GLU : GLY : LEU : SER : CYS : ARG : ARG : ARG : LEU : CYS : ARG : PRO : GLU : GLN : TRP : LEU : HIS :
   SER : TRP :*MET*: LYS : GLY : CYS : ARG : VAL : GLY : GLY : ASP : SER : ALA : GLY : GLN : SER : ASN : GLY : TYR : THR :
     ALA : GLY :**N**: ARG : ALA : VAL : VAL : SER : ALA : ALA : THR : LEU : GLN : ALA : ARG : ALA :*MET*: ALA : THR : PRO :
---------#---------#---------#---------#---------#---------#---------#---------#---------#---------#---------#---------#
 LEU : GLN : ILE : PHE : PRO : GLN : ARG : THR : PRO : PRO : SER : GLU : ALA : PRO : TRP : LEU : LEU : PRO :**N**: VAL :
   ALA : PRO : HIS : LEU : ALA : THR : THR : ASP : ALA : ALA : VAL : ARG : CYS : ALA : LEU : ALA : ILE : ALA : VAL : GLY :
     SER : SER : SER : PRO : SER : ASP : HIS : ARG : ARG : ARG : SER : GLN : LEU : GLY : SER : CYS : HIS : SER : CYS : ARG :



            790               800               810               820               830               840
            '                 '                 '                 '                 '                 '
CGAGCCGGATCC
GCTCGGCCTAGG

 ARG : ALA : GLY :
   GLU : PRO : ASP :
     SER : ARG :
---------#---------#---------#---------#---------#---------#---------#---------#---------#---------#---------#---------#
 SER : GLY : SER :
   LEU : ARG :
     ALA : PRO :
```

Figure 26. Hydropathy analysis of the aryletherase gene nucleotide sequence.

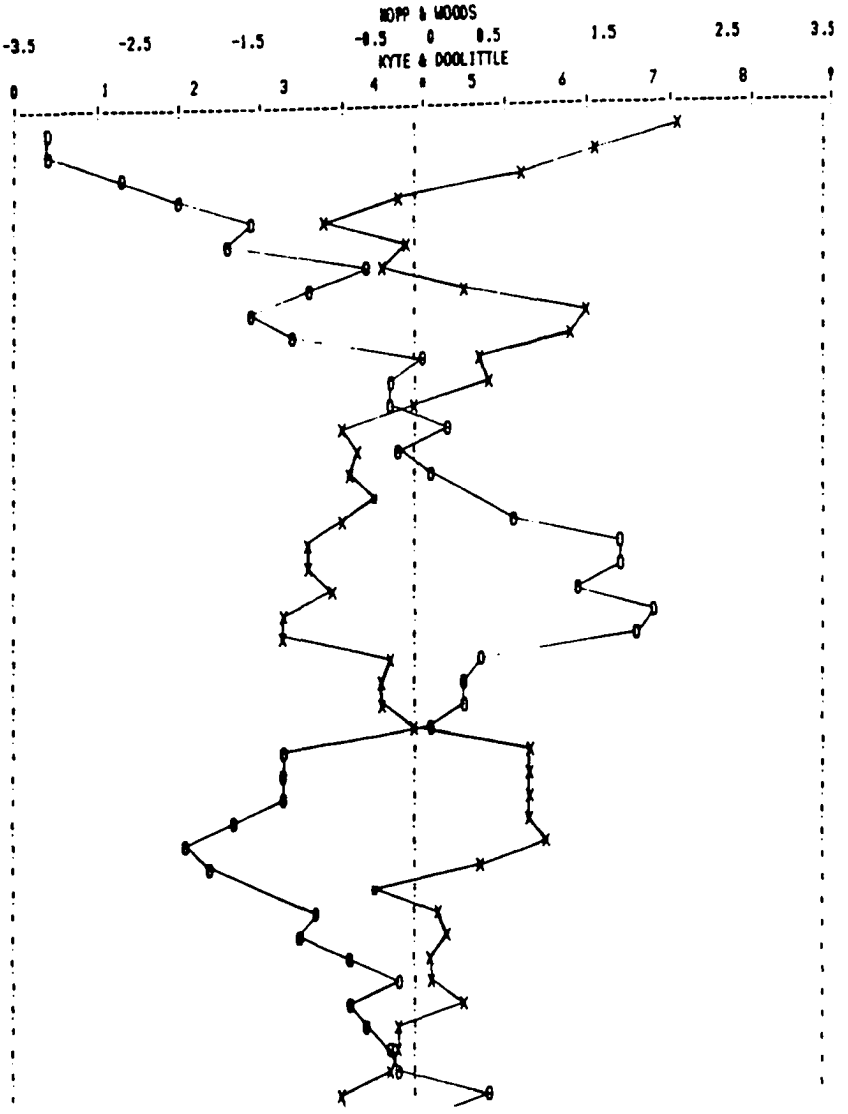
X -- HOPP & WOODS PARAMETER

0 -- KYTE & DOOLITTLE'S PARAMETER

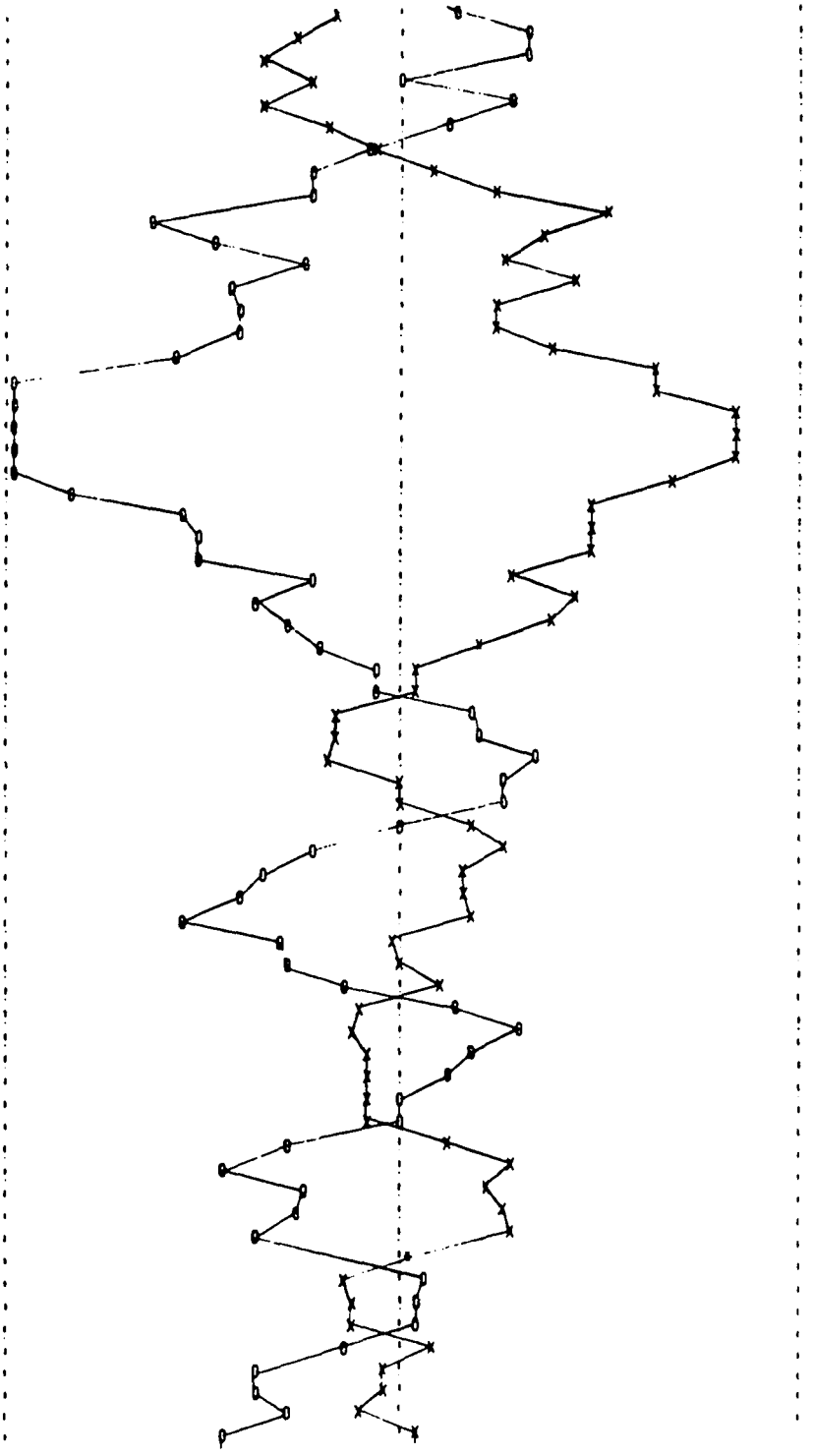
PROTEIN LENGTH = 175

ANALYSIS LENGTH = 5

COUNT		AMINO ACID	AVE. HW	AVE. KD
1	E	G.L.U	2.3	.46
2	R	A.R.G	1.6	.52
3	R	A.R.G	.92	1.28
4	R	A.R.G	- .14	1.92
5	N	N.I.S	- .74	2.74
6	N	N.I.S	- .84	2.48
7	T	T.N.R	- .24	3.96
8	Y	T.Y.R	.44	3.4
9	G	G.L.Y	1.5	2.76
10	R	A.R.G	1.4	3.2
11	V	V.A.L	.6	4.6
12	E	G.L.U	.7	4.26
13	R	A.R.G	0	4.32
14	A	A.L.A	- .6	4.9
15	C	C.Y.S	- .44	4.36
16	C	C.Y.S	- .54	4.7
17	H	H.I.S	- .34	4.12
18	P	P.R.O	- .6	5.66
19	S	S.E.R	- .9	6.02
20	V	V.A.L	- .9	6.02
21	G	G.L.Y	- .7	6.34
22	I	I.L.E	-1.06	7.16
23	V	V.A.L	-1.06	7
24	S	S.E.R	- .16	5.26
25	A	A.L.A	- .22	5.12
26	L	L.E.U	- .22	5.12
27	L	L.E.U	.04	4.74
28	R	A.R.G	1	3.1
29	P	P.R.O	1	3.1
30	A	A.L.A	1	3.1
31	A	A.L.A	1.02	2.6
32	R	A.R.G	1.18	2.06
33	R	A.R.G	.42	2.26
34	P	P.R.O	- .34	4.06
35	T	T.N.R	.26	3.48
36	S	S.E.R	.34	3.3
37	N	A.S.N	.18	3.84
38	I	I.L.E	.2	4.36
39	R	A.R.G	.46	3.82
40	P	P.R.O	- .1	4.02
41	A	A.L.A	- .1	4.26
42	S	S.E.R	- .2	4.48
43	A	A.L.A	- .62	5.32



45	G	G.L.Y	- .5	5.16
46	C	C.Y.S	- .86	5.98
47	L	L.E.U	-1.16	6.02
48	P	P.R.O	- .76	4.58
49	S	S.E.R	-1.12	5.0
50	L	L.E.U	- .58	5.08
51	F	P.N.E	- .16	4.16
52	N	A.S.N	.34	3.54
53	I	I.L.E	.9	3.54
54	R	A.R.G	1.86	1.74
55	S	S.E.R	1.32	2.46
56	G	G.L.Y	.96	3.48
57	D	A.S.P	1.56	2.66
58	R	A.R.G	.84	2.72
59	S	S.E.R	.86	2.72
60	V	V.A.L	1.4	2
61	R	A.R.G	2.3	.26
62	N	N.I.S	2.3	.26
63	R	A.R.G	3	0
64	R	A.R.G	3	0
65	R	A.R.G	3	0
66	R	A.R.G	2.4	.82
67	R	A.R.G	1.7	2.08
68	R	A.R.G	1.7	2.28
69	R	A.R.G	1.7	2.28
70	G	G.L.Y	1	3.54
71	A	A.L.A	1.6	2.92
72	D	A.S.P	1.34	3.3
73	R	A.R.G	.74	3.68
74	A	A.L.A	.14	4.26
75	D	A.S.P	.14	4.26
76	L	L.E.U	- .56	5.32
77	P	P.R.O	- .5	5.42
78	P	P.R.O	- .6	6.1
79	A	A.L.A	0	5.72
80	A	A.L.A	0	5.72
81	V	V.A.L	.7	4.58
82	A	A.L.A	.92	3.6
83	E	G.L.U	.56	2.98
84	A	A.L.A	.56	2.78
85	K	L.Y.S	.66	2.1
86	T	T.H.R	- .04	3.24
87	Y	T.Y.R	.04	3.3
88	R	A.R.G	.4	3.92
89	P	P.R.O	- .3	5.18
90	A	A.L.A	- .4	5.86
91	G	G.L.Y	- .24	5.32
92	A	A.L.A	- .24	5.08
93	A	A.L.A	- .22	4.58
94	A	A.L.A	- .22	4.58
95	S	S.E.R	.48	3.32
96	P	P.R.O	1.02	2.6
97	T	T.H.R	.82	3.42
98	A	A.L.A	.96	3.38
99	R	A.R.G	.98	2.88
100	R	A.R.G	.88	4.62
101	C	C.Y.S	- .48	4.82
102	S	S.E.R	- .38	4.68
103	T	T.H.R	- .38	4.68
104	V	V.A.L	.3	3.92
105	Q	G.L.N	- .08	2.9
106	A	A.L.A	- .08	2.9
107	S	S.E.R	- .34	3.28
108	R	A.R.G	.2	2.56



111	L	L.E.U	.74	3.72
112	R	A.R.G	1.7	2.08
113	R	A.R.G	1.7	2.08
114	A	A.L.A	1	3.34
115	G	G.L.Y	1.1	2.9
116	R	A.R.G	1	3.34
117	R	A.R.G	.1	5.08
118	A	A.L.A	- .44	5.28
119	G	G.L.Y	- .3	4.74
120	A	A.L.A	.3	3.92
121	V	V.A.L	.4	3.24
122	Q	G.L.N	.7	2.08
123	S	S.E.R	1.26	2.08
124	R	A.R.G	1.2	1.94
125	P	P.R.O	.5	3.2
126	P	P.R.O	.5	3.44
127	D	A.S.P	1.1	3.86
128	P	P.R.O	.5	3.68
129	A	A.L.A	.2	4.84
130	G	G.L.Y	.36	4.3
131	E	G.L.U	.36	4.86
132	G	G.L.Y	- .34	5.12
133	V	V.A.L	.26	4.5
134	S	S.E.R	.56	3.34
135	P	P.R.O	.5	3.44
136	A	A.L.A	1.1	3.86
137	E	G.L.U	1.8	1.8
138	P	P.R.O	1.8	1.6
139	G	G.L.Y	1.44	2.66
140	D	A.S.P	.98	2.48
141	R	A.R.G	.02	3.92
142	R	A.R.G	- .54	4.12
143	L	L.E.U	-1.14	4.7
144	Y	T.Y.R	- .78	3.88
145	L	L.E.U	- .42	4.98
146	Q	G.L.N	- .36	4.6
147	P	P.R.O	.2	4.6
148	G	G.L.Y	.24	4.22
149	V	V.A.L	- .22	4.84
150	A	A.L.A	- .22	4.84
151	D	A.S.P	.48	2.98
152	Q	G.L.N	- .42	4.22
153	Y	T.Y.R	-1.02	5.66
154	V	V.A.L	- .44	6.28
155	D	A.S.P	.24	4.74
156	F	P.H.E	- .36	5.36
157	L	L.E.U	- .36	5.36
158	A	A.L.A	- .1	3.98
159	D	A.S.P	- .3	4.46
160	G	G.L.Y	-1.2	6
161	F	P.H.E	-1.28	5.94
162	N	N.I.S	- .78	5.08
163	V	V.A.L	- .44	5.02
164	V	V.A.L	- .3	3.48
165	T	T.H.R	.6	1.86
166	P	P.R.O	.68	1.92
167	Q	G.L.N	.72	1.54
168	Q	G.L.N	.32	2.98
169	K	L.Y.S	.28	3.36
170	G	G.L.Y	.28	3.24
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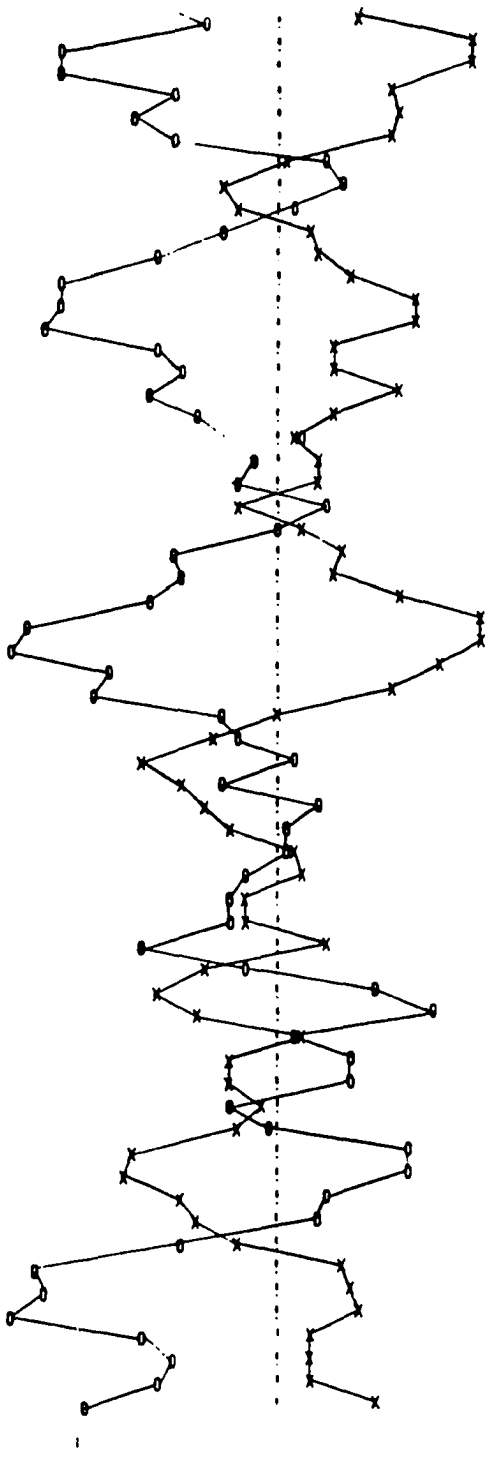
172	L	L.E.U		
173	P	P.R.O		
174	R	A.R.G		



DISCUSSION

Recombinant DNA techniques were utilized in the present study to successfully clone a gene encoding aryletherase activity from an Erwinia sp. into Escherichia coli Cs412 using pBR322 as a vector. Initial studies by Y. Chon (8) demonstrated that the Erwinia sp. possesses the enzymatic capability to degrade lignin to lower molecular weight aromatic compounds. Lignin model compounds containing arylether bonds were shown to be degraded by oxidation, demethoxylation, and ring fission processes. Therefore, excision of the gene encoding the aryletherase from the Erwinia sp. genome and expression in E. coli allows for further examination of this biocatalyst in the absence of ring fission and other catabolic enyzmes.

The successful cloning of the aryletherase gene was enabled by the novel utilization of salicin as a selective carbon source and p-nitrophenyl-β-D-glucopyranoside (PNPG) as a chromogenic substrate for the detection of enzyme activity. The high molecular weight, heteropolymeric, aromatic structure of lignin precludes its use as a sole carbon source for the selection of an E. coli transformant carrying the desired gene. Salicin was chosen as the selective growth substrate based on its structural similarities to arylether-containing model compounds. Salicin is a glucoside with salicyl alcohol as an aglycol. This molecular configuration bears structural similarity to arylether bonds, and can therefore be thought of as a pseudoether bond.

The Erwinia sp. is able to utilize salicin as a sole carbon source but, fortuitously, lacks the ability to grow on cellobiose or lactose, i.e. lacks demonstratable cellobiase and lactase activities. Thus, it

was thought that the Erwinia sp. possesses an enzyme which recognizes molecules containing aromatic nuclei. Further, E. coli Cs412 host cells are unable to grow on salicin and also lack β-glucosidase activity. Therefore, growth of E. coli cells transformed with Erwinia sp. DNA shotgun cloned into pBR322 on salicin minimal media depended on the transfer of a gene encoding an enzyme which can recognize and cleave salicin to release glucose as a carbon and energy source for utilization by the E. coli host. This strategy, coupled with further screening for aryletherase activity using PNPG ultimately led to the selection of two clones for further study.

Attempts to detect aryletherase activity from Erwinia sp. genomic libraries constructed in λ phage EMBL4 and plated in the presence of x-glu were unsuccessful. Low expression of enzyme activity preceeding cell lysis is one possible explanation for failure to detect the release of the chromogen. Also, the phage promoter, P_L, has been deleted in recombinant derivatives of EMBL4. Therefore, expression of genes cloned in EMBL4 in E. coli depends on utilization of cloned promoters.

Lignin model compounds have been utilized extensively in past studies to examine the metabolic capabilities of various microorganisms in relation to lignin biodegradation. In the present study, vanillin, vanillic acid, syringaldehyde, and syringic acid were used as lignin models based upon chemical similarities with lignin substructures and also because these compounds contain methoxyl groups linked via ether bonds to the aromatic moiety. E. coli Cs412 transformants harboring either pNC1 or pNC2 expressed aryletherase activity, which is oxidative. Vanillin was initially oxidized to vanillic acid followed by further

degradation to catechol. In analagous manner, syringaldehyde was oxidized to syringic acid. Apparently the arylether ase from the Erwinia sp. is able to act upon a variety of aromatic compounds. Conversion of vanillin to catechol suggests that this enzyme is involved in preparing the aromatic ring for subsequent ring fission processes. Degradation of p-phenoxyphenol by the clones demonstrates further the ability of this enzyme to cleave arylether bonds. Most interestingly, the cloned enzyme was shown to be active on kraft lignin, releasing at least one lower molecular weight aromatic intermediate from the lignin polymer in just 2 hours incubation time.

It is interesting to note that recent reports of β-O-4 ether cleavage of model compounds by Streptomyces viridosporus produces intermediates which are also degraded through vanillin and vanillic acid (20). In recent studies in our laboratory, nick-translated pNC1 was used to probe a number of sewage organisms in colony hybridizations for homologous DNA sequences. Of six colonies showing hybridization to the probe, four organisms were subsequently demonstrated to be capable of growth with lignin as the sole carbon source. These organisms appear to be different from the Erwinia sp. These results suggest that organisms capable of decomposing at least some substructural components of lignin may actually be quite prevalent in nature and are not confined to members of wood-rotting organisms.

Analysis of one plasmid (pNC1) carrying the arylether ase gene at the DNA level revealed an insert of approximately 810 bp of Erwinia sp.

genomic DNA as determined by agarose gel electrophoresis. DNA sequence data showed this insert to be 792 bp in length. Thus, these independently derived results differ only about 2%.

Anomalous results were obtained in attempting to characterize the second clone exhibiting aryletherase activity. Both restriction enzyme analyses and hybridization studies were inconclusive in determining the nature of pNC2 beyond its DNA sequence homology with pNC1.

DNA sequence data indicates pNC1 contains the aryletherase gene lacking a consensus promoter sequence. Expression of the gene in E. coli Cs412 is apparently the result of readthrough of transcription initiated at the tetracycline resistance gene promoter of the pBR322 vector. The physical map of pNC1 indicates that the gene is properly oriented with respect to tetracycline resistance gene transcription for readthrough to occur. It will be interesting to examine DNA regions upstream from the aryletherase gene for promoter-like sequences. Experiments to isolate clones containing regulatory regions of the aryletherase gene from phage libraries are currently in progress in our laboratory.

Unsuccessful attempts at obtaining increased levels of expression of the aryletherase gene in the expression vector pCQV2 were explained upon examination of the DNA sequence data. Insertion of the gene at the Bam HI site of pCQV2 was not in the proper translational reading frame with respect to the translational start site carried by the vector. Experiments are in progress to place the aryletherase gene under control of the lambda promoter, PL, in the expression vector pGW7 (27).

Translational analysis of the DNA sequence has identified a putative translational start site 21 bases from the Bam HI end of the fragment carrying the aryletherase gene. The DNA sequence at -10 from the AUG initiation codon is UAGGA. This sequence bears strong homology with Shine-Delgarno (ribosome-binding) sequences examined in other procaryotic systems (36).

The initiation codon is followed by an open reading frame with coding capacity for 175 amino acids. This corresponds to a protein with an estimated molecular weight of about 21,000 daltons.

In the Erwinia sp., as well as in E. coli Cs412 transformants carrying pNC1, enzymatic activity can be detected in whole cells, suggesting that the aryletherase may be localized in the periplasm or loosely bound to the cytoplasmic membrane. Hydropathy analysis of the predicted translation product reveals two striking features: an amino terminus of 12 amino acids that is basic in nature which is followed by a hydrophobic region 15 amino acids in length. Such characteristics are similar to those which have been observed in periplasmic and membrane bound proteins in E. coli (87). It remains to be determined whether these protein domains are important in the localization of the aryletherase gene product.

To date, the only ligninolytic enzyme characterized in detail has been reported by Tien and Kirk (90, 91). This enzyme is active against $C\alpha$-$C\beta$ bonds in the propane side chains of lignin. This study represents advances in the characterization and study of the lininolytic system present in a saprophytic Erwinia sp. More specifically, an enzyme has been cloned which is active in breaking arylether bonds in lignin model

compounds. Lignin represents a grossly underutilized renewable resource which could conceivably be processed to yield valuable chemical feedstocks. Manipulation of the Erwinia sp. aryletherase gene by recombinant DNA techniques has made it possible to release at least one lower molecular weight aromatic product from kraft lignin. The enzyme has also been shown to effect the conversion of vanillin to catechol without further ring cleavage. Use of a single enyzme system such as this may contribute to the development of processes in which aromatic compounds can be economically produced from lignin.

The pNC1-lacZ construct was designed to make use of an inexpensive, selective growth substrate such as whey to ensure maintenance of plasmids encoding aryletherase in E. coli host strains. The use of ampicillin to force plasmid maintenance is not considered to be economically feasible on an industrial scale.

Should the aryletherase prove to be stable as a membrane bound or periplasmic enzyme in E. coli transformants, immobilized cell reactors may be an approach to consider for the biocatalytic conversion of lignin to lower molecular weight aromatic intermediates. In light of the antibiotic properties of some aromatic lignin components (98), such a design would allow the removal of growth inhibitory compounds from the reactor as lignin decomposition proceeds.

Finally, the aryletherase may be developed as part of a biomechanical pulping process. Pretreatment of lignocellulosic materials with the enzyme may lessen the thermochemical demand required to achieve pulping and result in savings in the cost of energy and chemicals to the pulping industry.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Microbiology

Title of Dissertation: Studies on the Molecular Cloning of a Gene for Aryletherase from a Ligninolytic Erwinia sp.

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